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POSITIONAL REGULATION OF B LYMPHOCYTE DEVELOPMENT IN BONE MARROW

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Positional Regulation of B Lymphocyte Development in Bone Marrow

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"Regardez le ciel. Demandez-vous: le mouton oui ou non a-t-il mangé la fleur? Et vous verrez comme tout change...

Et aucune grande personne ne comprendra jamais que ça a tellement d'importance!"

- Antoine de Saint-Exupéry in *Le petit prince*, 1946

These concluding lines of *The Little Prince* encourage us (the readers) to think about what we have just learnt. I cannot measure how much I have grown up, as a scientist and as a person during my doctoral studies. The elaboration of the present thesis was an adventure, not always enjoyable but always very challenging, and **instructing**. As this thesis reaches its end, I realize the most important lesson was to never stop questioning, and improving myself. The last five years would not have been possible without the support of several people.

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Por fim, dedico esta tese ao meu falecido avô Manuel António Cordeiro.

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List of abbreviations

AGM	Aorta-gonad-mesonephros
BCR	B cell receptor
Bglap	bone gamma-carboxyglutamic acid-containing protein gene
BLNK	B cell linker protein
BM	Bone marrow
BSA	Bovine serum albumin
BV	Brilliant Violet
CAR	CXCL12-abundant reticular
CB2	Cannabinoid receptor 2
CD	Cluster of Differentiation
CLP	Common lymphoid progenitor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
TdT	Deoxynucleotidyltransferase
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
E2A	Immunoglobulin enhancer-binding factors E12/E47
EBF	Early B cell Factor
ETP	Emigrant thymocyte progenitors
FBS	Fetal bovine serum
FLT3	Fms-like tyrosine kinase 3
FLT3L	Fms-like tyrosine kinase 3 ligand
FOB	Follicular B cell

FOXC1	Forkhead box protein C1
FOXO1	Forkhead box protein O1
FOXO3	Forkhead box protein O3
GAL1	Galectin-1
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor)
GMP	Granulocyte and monocyte progenitors
GPCR	$G\alpha_i$ protein-coupled receptors
HBSS	Hank's balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HSC	Hematopoietic Stem Cells
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IL-7R	Interleukin-7 receptor
ILC	Innate lymphoid cell
IRF4	Interferon regulatory factor 4
IVM	Intravital microscopy
JAK	Janus Kinase
KLF2	Krüppel-like factor-2
KO	Knock out
LEPR	Leptin Receptor
LPS	Lipopolysaccharide
LT	Lymphotoxin

LTi	Lymphoid tissue induced cells
LY6D	Lymphocyte Antigen 6 Complex, Locus D
MB-1	B-cell antigen receptor complex-associated protein alpha chain
MBZ	Marginal B zone cell
MCL1	Myeloid-cell leukemia sequence 1
M-CSF	Macrophage colony-stimulating factor
MEP	Megakaryocyte and erythroid progenitors
MPP	Multi-potent progenitor
MSC	Mesenchymal stem cell
MSPC	Mesenchymal stem and progenitor cell
Mx-1	Myxovirus resistance-1
MZ	Marginal zone
NK	Natural killer
OCT	Optimal Cutting Temperature
O.N.	Overnight
Osx	Osterix
PAX	Pair box transcription factor family
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGFRα	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-phosphate
Prx-1	Pair related homeobox-1 gene
PTH	Parathyroid hormone
PTX	Pertussis toxin
RAG	Recombination-activating gene
RANK	Receptor activator of nuclear factor kappa-B

RANKL	Receptor activator of nuclear factor kappa-B ligand
RBC	Red blood cell
RPMI	Roswell park memorial institute
RT	Room temperature
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine 1 phosphate receptor
SCF	Stem cell factor
SDF-1	Stromal-cell derived factor-1
SLO	Secondary lymphoid organ
STAT	Signal Transducer and Activator of Transcription
SYK	Spleen tyrosine kinase
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule 1
WT	wild-type
X-SCID	X-linked severe combined immunodeficiency
YFP	Yellow fluorescent protein

Abstract

Hematopoietic stem cells (HSC) are maintained throughout life in specialized niches in bone marrow (BM). The hematopoietic niches are suggested to support the maintenance and differentiation of hematopoietic progenitor cells. It is still unknown whether and how the niche contributes to lymphocyte differentiation. The CXCR4/CXCL12 pathway is fundamental for positioning of HSC within the hematopoietic niches, and therefore is a potential candidate to mediate chemoattraction to regulatory cues of lineage differentiation. Deficiencies in CXCR4 affect hematopoiesis, and specifically compromise B lymphopoiesis. Whether CXCR4 is required at early progenitor stages for differentiation or only required for HSC quiescence has not been addressed. Here, we conditionally deleted CXCR4 at different hematopoietic checkpoints, and found that CXCR4 was critically required at the MPP and CLP stage for cell positioning in proximity to rare IL-7-expressing cells. Moreover, this close positioning is critical for achieving sufficient IL-7R signaling. Deficiency in CXCR4 compromises the close positioning of CLP relative to IL-7-expressing cells, indicating that CXCR4 chemoattraction is fundamental for IL-7 consumption at this stage and that IL-7 producers in bone marrow are components of the B cell differentiation niche. We further showed that IL-7 is expressed by a heterogeneous population of mesenchymal progenitor cells, which express Leptin receptor, and differentiated into adipocytes and osteoblasts in vivo.

After the commitment to B lymphocyte lineage, CXCR4 is required for the retention of immature B cells in bone marrow parenchyma. We found that a twofold down-regulation of CXCR4 together with a GPCR-independent mechanism is critical for B cell egress into the periphery, where they complete their differentiation pathway. The passive mode of BM cell egress also contributes significantly to the export of NK cells.

Collectively, our findings provide a groundbreaking evidence of the overlapping between HSC maintenance and lymphoid differentiation niches, and are a valuable contribution to the understanding of B lymphopoeisis in vivo.

Resumo

Na medula óssea, nichos especializados são responsáveis pela manutenção e diferenciação das células estaminais hematopoiéticas. Pouco ainda se sabe sobre a intervenção do nicho na diferenciação das células estaminais. O receptor CXCR4 e o seu ligando CXCL12 são cruciais para o posicionamento das células estaminais hematopoieticas nos referidos nichos e daí serem potenciais candidatos na mediação da quimoatracção dos progenitores durante a diferenciação hematopoietica. A deficiência em CXCR4 compromete a hematopoiese, sobretudo o desenvolvimento de linfócitos B. Desconhece-se se o CXCR4 atua nas células estaminais hematopoiéticas ou se é fundamental a nível dos progenitores. A deleção de CXCR4 em diferentes etapas da hematopoiese foi a estratégia escolhida para responder a esta questão. Progenitores multipotentes e progenitores de linfócitos dependem de CXCR4 para se posicionarem perto de células produtoras de IL-7. A deficiência em CXCR4 compromete o posicionamento e o consumo de IL-7, indicando que as células produtoras de IL-7 são importantes constituintes do nicho de diferenciação de linfócitos B. Este estudo também revela que uma população heterogénea de progenitores do mesênquima produz IL-7, bem como CXCL12, expressa o receptor de Leptina e tem capacidade de diferenciação em adipócitos e osteoblastos.

Após entrada na via de desenvolvimento de linfócitos B, o CXCR4 retêm células B imaturas no parênquima da medula óssea. Para saírem da medula óssea para a circulação, as células B reduzem para metade a quantidade de CXCR4 que é expressa na superfície celular e são auxiliadas por um mecanismo independente de GPCR que as empurra para a circulação. Este mecanismo também contribui para a exportação de células NK.

Os resultados obtidos no âmbito desta tese alicerçam o modelo no qual o nicho de manutenção das células estaminais hematopoiéticas controla a diferenciação hematopoiética. Ademais, contribuem para a compreensão do desenvolvimento de linfócitos B in vivo.

Chapter 1

Introduction

Chapter 1 | Introduction

Hematopoiesis generates erythrocytes and the various types of leukocytes (**Figure 1.1**). Hematopoietic Stem Cells (HSC) are the only cells capable of generating all hematopoietic lineages throughout life, and have been characterized by two main characteristics, at single cell level(Cumano and Godin, 2007):

- multipotency, which is the capacity of a single cell to originate a differentiated progeny comprising different cell types;
- self-renewal, which allows maintenance of HSC pool.

HCS have been detected in several tissues throughout development. The first HSC arise in the aorta-gonad-mesonephros (AGM) region, and the yolk sac, followed by the placenta, fetal liver, spleen, and finally migrate to bone marrow (BM) right after birth(Cumano and Godin, 2007). Seminal work by Metcalf and Moore(Metcalf, 1970, Moore and Metcalf, 1970) and by Till and McCullough(Till, 1961) introduced the notion that multipotent progenitors found in adult BM are responsible for the active regeneration of the hematopoietic compartment.

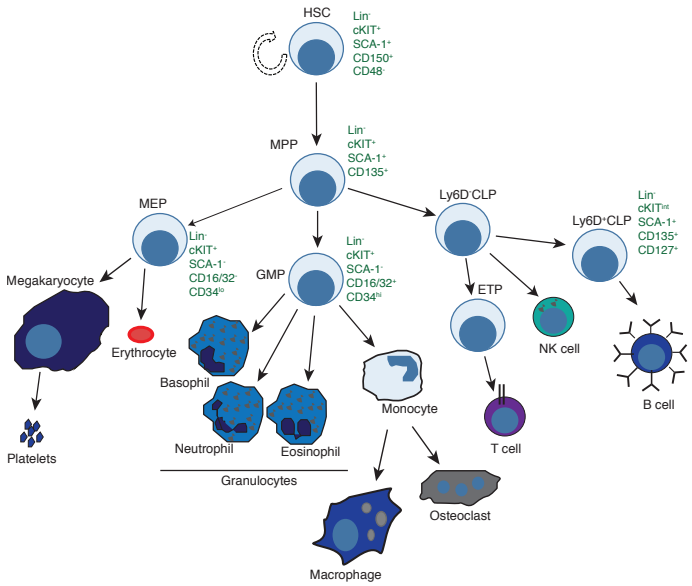

Figure 1.1| Scheme of murine adult hematopoiesis. Distinct hematopoietic stem and progenitor cell stages have been described by correlating surface markers expression (in green) and functional properties. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; NK, natural killer cell.

1. Anatomy of the Bone Marrow

The BM is a versatile organ, harboring not only most of the adult hematopoiesis but also osteogenesis. In addition to hematopoietic cells, BM also contains different types of cells. Mesenchymal stem cells (MSC) originate osteoblasts, adipocytes, and chondrocytes. Osteoprogenitors, osteoblasts, osteocytes support the bone formation. Other BM stromal cell populations, such as neuronal cells, glial cells, and adipocytes, may regulate hematopoiesis (Anthony and Link, 2014).

Within the BM parenchyma, there is a rich network of stromal cells, MSC and adipocytes interspersed between islands of hematopoietic cells (Anthony and Link, 2014). Osteoblasts and bone lining cells form a layer between mineralized bone and the bone marrow known as endosteum. Near the endosteum there is a rich supply of arterioles, as well as sinusoids (Morrison and Scadden, 2014). The BM of long (e.g., femur and tibia) and flat (e.g., calvaria) bones is highly vascular (Nombela-Arrieta et al., 2013, Sipkins et al., 2005). In long bones, central longitudinal arteries transform into radial arteries that in turn divide into arterioles near the endosteum (Nombela-Arrieta et al., 2013). Arteries feed into sinusoids, which in turn extend back towards the central cavity where they coalesce into a large central sinus to form the venous circulation (Anthony and Link, 2014, Morrison and Scadden, 2014). The venous sinusoids, which form a reticular network of fenestrated vessels, are thought to be the major sites of leukocyte egress from the bone marrow into the circulation (Campbell, 1972). Surrounding BM sinuses, adventitial reticular cells branch into the surrounding hematopoietic space (Lichtman, 1981, Weiss, 1976). B cells have been described to associate with this abundant type of BM stromal cell (Jacobsen and Osmond, 1990).

The positioning of hematopoietic stem and progenitor cells within BM is critical to understand the homeostasis, repair of the hematopoietic system, and as well to further clarify the architectural organization of the BM.

2. The Hematopoietic Stem Cell Niche Concept

Like all metazoan cells, HSC and hematopoietic progenitors require a supporting milieu. Dexter and colleagues demonstrated the dependence of HSC on supporting non-hematopoietic cells in BM (Dexter et al., 1977). Lord demonstrated the importance of the architectural organization of bone marrow to support hematopoiesis (Lord et al., 1975). Then, Raymond Schofield formally proposed the stem cell niche (Schofield, 1978).

The theory of Schofield postulated that hematopoietic stem cells were located in physical sites denominated niches (**Table 1.1** and **Figure 1.2**). The niche is an anatomical location that affects stem cell number and behavior by inducing self-renewal in proximity of this location or inducing differentiation at a distance (Schofield, 1978). In addition, the niche was capable of preventing the accumulation of genetic mutations during self-renewal (Schofield, 1978).

Table 1.1| Schofield's proposed features of a stem cell niche. Adapted from

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- Restriction on stem cell entry into cell cycle and differentiation programs
- Integration of signals reflecting tissue and organismal state
- Imposition of stem cell features on daughter cells
- Mechanism for limiting "mutational errors"

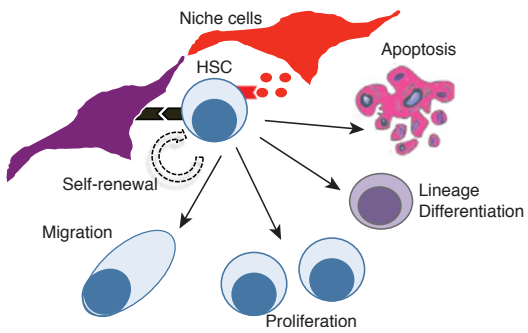

Figure 1.2| Functions of the hematopoietic niche. Through the production of several factors like cytokines and membrane-molecules, the niche instructs the cell-fate decision of HSC. Adapted from (Rieger and Schroeder, 2012).

Even though the niche theory has been around for several decades now, only about 10 years ago in vitro and in vivo studies have experimentally demonstrated that non-hematopoietic cells in BM are capable of regulating HSC (Taichman and Emerson, 1994, Calvi et al., 2003, Zhang et al., 2003). In the following sections I will cover the progress made in unveiling the localization and the cellular components of the HSC niche.

3. Mapping of HSC and their niches within BM

Analyses of different transgenic mice have implicated osteoblasts in the regulation of HSC in BM (Calvi et al., 2003, Zhang et al., 2003, Visnjic et al., 2004). HSC have also been suggested to interact with osteoblasts in the endosteum (Zhang et al., 2003, Arai et al., 2004, Wilson et al., 2004). However, several evidences indicate that the endosteum is unlikely to be the only niche for HSC. First, sites of extramedullary hematopoiesis, such as the liver, lack osteoblasts (Taniguchi et al., 1996). Second, cytokines readily mobilize HSC into circulation (Laterveer et al., 1995). Finally, HSC are motile and recirculate (Wright et al., 2001). Therefore, it became fundamental to identify unequivocally HSC in situ.

The positive staining for CD150 combined with negative staining for lineage markers, CD48 and CD41 is a hallmark in HSC biology because it allowed the identification of HSC in situ using a simple two color staining (Kiel et al., 2005). The majority of HSC in bone marrow and spleen were observed adjacent to sinusoidal vessels (Kiel et al., 2007, Kiel et al., 2005). Indeed, HSC are five times more likely than other hematopoietic cells to be localized close to a sinusoidal vessel (Kiel et al., 2007). Moreover, less than 20% of HSC are localized within 10 μm of the endosteum (Kiel et al., 2007, Kiel et al., 2005, Lo Celso et al., 2009). These observations suggested that HSC might be maintained in a perivascular niche. Still, it is possible that HSC near vessels were in transit. Therefore, the niche cellular components have to be further characterized.

4. Dissecting the elements of the HSC niche

The complexity of the anatomy and cellular composition of the HSC maintenance niche has increased over the last decade, in part due to the heterogeneity of HSC and the fact that several stromal cells in BM seem to exhibit HSC maintenance properties (**table 1.2**, on next page). Despite early reports implying osteoblasts in the regulation of HSC maintenance niche (Calvi et al., 2003, Zhang et al., 2003, Visnjic et al., 2004, Arai et al., 2004, Wilson et al., 2004), it is currently accepted that osteoblasts do not directly regulate HSC, but support B lymphopoiesis (Taichman et al., 1996, Taichman, 2005, Visnjic et al., 2004, Zhu et al., 2007, Ding and Morrison, 2013b). Mesenchymal stem and progenitors cells (MSPC), which are multipotent cells capable of differentiating into osteoblasts, chondrocytes, adipocytes and stromal cells (Frenette et al., 2013), have been described as critical niche cells (see below)(Mendez-Ferrer et al., 2010b, Kunisaki et al., 2013, Pinho et al., 2013, Morrison et al., 1995, Ding et al., 2012, Ding and Morrison, 2013b, Zhou et al., 2014, Nagasawa et al., 1991, Sugiyama et al., 2006, Omatsu et al., 2010, Greenbaum et al., 2013, Omatsu et al., 2014). Mature hematopoietic cells also play a role in forming the hematopoietic niche. Megakaryocytes secrete the chemokine CXCL4, which controls HSC cell cycle activity (Bruns et al., 2014). $CD169^{+}$ macrophages, besides being a key components for medullary erythropoiesis (Chow et al., 2013), also regulate the hematopoietic niche by cross-talking with MSPC, inducing the expression of CXCL12 and thus promoting the retention of HSC and progenitors in the hematopoietic niche (Chow et al., 2011). Sympathetic nerves enwrap MSPC and secret noradrenaline in a circadian-manner, which is sensed by MSPC and causes them to reduce the expression of CXCL12, thus enhancing the BM egress of HSC and progenitors (Mendez-Ferrer et al., 2008, Mendez-Ferrer et al., 2010a). Besides demonstrating the diversity of cells supporting HSC maintenance, these studies also support that MSPC regulate hematopoietic differentiation in response to environmental cues. We have recently reviewed how hematopoietic progenitors and niche supporting cells sense and respond to stress cues, suggesting a potential role for the hematopoietic niche in the development pathologies such as rheumatoid arthritis and anemia of chronic infections (see appendix).

Table 1.2| BM populations of the HSC niche. Adapted from [(Ugarte and Forsberg, 2013)].

Cell Type	Markers	Mouse Model [a]	Niche function
Perivascular Stromal cell - Nestin+ cells - SCF+ cells - CXCL12-abundant cells (CAR)	CXCL12^{hi} SCF^{hi} NG2 Pdgfr-a Pdgfr-b CD146 Sca-1 VCAM1 CFU-F potencial Mesenchymal lineage differentiation	*Nestin*-GFP (Mendez-Ferrer et al., 2010b, Ding et al., 2012) *Prx1*-cre (Ding and Morrison, 2013b, Greenbaum et al., 2013) *Cxcl12*-GFP (Sugiyama et al., 2006)/ DsRed (Ding and Morrison, 2013b) *Cxcl12*-DTR-GFP (Omatsu et al., 2010) *Lepr*-cre (Ding and Morrison, 2013b, Ding et al., 2012) *Scf*-GFP (Ding et al., 2012)	HSC maintenance and retention in BM Direct supply of soluble and non-soluble factors for HSC Precursors of other niche cells – osteoblasts, adipocytes, chondrocytes, etc.
Sinusoidal endothelial cells	CD31 Endomucin VE-cadherin VCAM1 Laminin Sca-1 MECA-32 Endoglin	*Tie2*-cre (Ding and Morrison, 2013b, Ding et al., 2012, Greenbaum et al., 2013) *Cdh5*-CreER (Wang et al., 2013)	HSC maintenance
Osteoprogenitors	Osterix Runx2 CD146	*Osx*-cre (Greenbaum et al., 2013) *Prx1*-cre (Greenbaum et al., 2013, Ding and Morrison, 2013b)	Lymphoid progenitor cell niche
Osteoblasts (OB)	Osteocalcin Osteopontin ALPL N-cad CXCL12^{low}	*Prx1*-cre (Greenbaum et al., 2013, Ding and Morrison, 2013b) *Col2.3*-cre (Ding and Morrison, 2013b, Ding et al., 2012) *Osteocalcin*-cre (Greenbaum et al., 2013) *Osx*-cre (Greenbaum et al., 2013, Ding and Morrison, 2013b)	Lymphoid progenitor cell niche
Adipocytes	Fabp4 Adiponectin Perilipin	A-ZIP/F1 (Naveiras et al., 2009)	Negative regulators of hematopoiesis
Monocytes/ Macrophages	Gr-1 CD169 CD11b F4/80	*Cd169*-DTR (Chow et al., 2011) *Gr1*-DTR (Chow et al., 2011) MAFIA (Winkler et al., 2010, Chow et al., 2011) Clodronate liposomes (Winkler et al., 2010, Chow et al., 2011) CD68:G-CSFR (Christopher et al., 2011)	Regulating MSC and OB function. Active player in G-CSF mobilization.
Non-myelinating Schwann cells	Active TGF-B GFAP Nestin	Tgfbr2$^{fl/-}$ (Yamazaki et al., 2011)	Maintenance of HSC quiescence
Regulatory T cells	CD4^{+}CD25^{+}Foxp3^{+}	*Foxp3*^{GFP} (Fujisaki et al., 2011) *Foxp3*-GFP-DTR (Yamazaki et al., 2011)	Immune protection to the HSC niche

[a] Mouse models used to determine the role of the respective cell population on the HSC niche.

The following paragraphs focus on the principal studies and the experimental approaches used to identify non-hematopoietic niche cells. The niche cells must meet the following criteria: 1) be a rare population in BM; 2) harbor HSC and hematopoietic progenitors close by; 3) produce HSC maintenance factors, which are important regulators of the HSC niche; and 4) capable of sensing and responding accordingly to systemic and local cues in order to maintain an optimal hematopoietic output (Frenette et al., 2013). For the purpose of elucidating the niche cells and its molecular regulators, some studies focused on the extracellular signals that maintain and regulate HSC, while others attempt to identify the cells producing such factors. The conditional deletion of key factors in restricted cell populations using *Cre* recombinase expressed under the control of (in principle) cell type-specific promoters and the improvement in BM imaging techniques have become key tools to determine whether a single factor used by a candidate cell type is critical for the maintenance of HSC and hematopoietic progenitors.

CXC chemokine ligand 12 (CXCL12) and Stem Cell Factor (SCF) are critical for the maintenance of HSC within their niche, and their sources in BM are potential constituents of the HSC niche. One of the first attempts to identify the cellular constituents of the HSC niche was to identify the sources of CXCL12 in BM. CXCL12 (also known as stromal cell-derived factor-1, SDF-1) was first isolated from stromal cell lines used in in vitro B lymphocyte development assays (Tashiro et al., 1993, Nagasawa et al., 1994), and was first characterized as a growth-stimulating factor for a stromal cell-dependent B cell precursor clone (Nagasawa et al., 1994). Nonetheless, CXCL12 and its receptor CXCR4 (Nagasawa et al., 1994, Nagasawa et al., 1996, Bleul et al., 1996, Oberlin et al., 1996, Tachibana et al., 1998, Zou et al., 1998, Ma et al., 1998) are involved in a variety of important processes, like the colonization of BM by hematopoietic cells, namely HSC, during ontogeny (Nagasawa et al., 1996, Tachibana et al., 1998, Ma et al., 1998, Ara et al., 2003b), the intestinal angiogenesis, and the development of the cerebellum (Nagasawa et al., 1996, Tachibana et al., 1998, Zou et al., 1998, Ma et al., 1998). CXCR4 signaling also retains hematopoietic cells in primary lymphoid organs (Ma et al., 1999). Within the HSC niche, CXCL12 and CXCR4 regulate the retention and quiescence of HSC (Greenbaum et al., 2013, Ding

and Morrison, 2013b, Schajnovitz et al., 2011, Nie et al., 2008, Sugiyama et al., 2006).

Using *Cxcl12*$^{GFP/+}$ reporter mice, Nagasawa's group identified a population of perivascular reticular cells, which were denominated as CXCL12-abundant reticular cells (CAR cells). CAR cells were observed in close proximity with HSC in situ, suggesting that the CAR cell population were niche cells (Sugiyama et al., 2006). In a follow-up study, depletion of CAR cells using a diphtheria toxin inducible system led to HSC loss, indicating that the CAR cell population contained cells with niche activity. Moreover, CAR cells produce most of the CXCL12 (and SCF, see below) in BM, exhibited multipotent progenitor activity, and showed osteoblastic and adipocytic lineage differentiation potential in vivo (Omatsu et al., 2010). The transcription factor FOXC1 inhibits adipogenic differentiation in CAR cells, which is critical for the maintenance of HSC and hematopoietic progenitors. This transcription factor is also involved in up-regulating CXCL12 and SCF. Therefore, FOXC1 is a specific transcriptional regulator essential for the development and maintenance of mesenchymal niche for hematopoietic cells and progenitors (Omatsu et al., 2014). Around the same time, Frenette's lab identified Nestin-expressing mesenchymal stem cells (MSC) as components of the HSC niche (Mendez-Ferrer et al., 2010b). Nestin^{+} MSC have been observed in close contact with HSC and express HSC maintenance genes like *Cxcl12* and *Scf* (Mendez-Ferrer et al., 2010b).

In 2012, Morrison and colleagues focused on the BM sources of SCF. SCF, whose receptor is cKIT, is required for HSC maintenance in vivo (Czechowicz et al., 2007, Broudy, 1997, Russell, 1979, Ogawa et al., 1991). Membrane-bound SCF is critical for HSC maintenance by acting locally to form a niche (Wolf, 1978, Barker, 1994, Barker, 1997). Perivascular and endothelial cells are the major SCF producers in BM (Ding et al., 2012). Profiling of SCF-expressing cells revealed that SCF cells highly express *Lepr*. Conditional deletion of *Scf* in endothelial cells or leptin receptor (*Lepr*)-expressing perivascular stromal cells depleted the HSC pool, indicating that perivascular *Lepr*$^{+}$ stromal cells are niche cells. Furthermore, this study demonstrated that the HSC niche is perivascular (Ding et al., 2012).

Lepr^{+} cells have been shown to be mesenchymal progenitor cells with osteoblastic and adipocytic lineage potential (Zhou et al., 2014). This is in agreement with the observation that Nestin-expressing mesenchymal stem cells (MSC) are constituents of the HSC niche (Mendez-Ferrer et al., 2010b). In addition, perivascular *Lepr*$^{+}$ cells and Nestin^{+} reticular MSC localize largely together in the bone marrow (Kunisaki et al., 2013, Pinho et al., 2013). Noteworthy, SCF is not expressed in bone-lining osteoblasts, indicating that osteoblasts are not an essential source of SCF for HSC maintenance (Ding et al., 2012).

A year later, another study from the Morrison's group demonstrated that CXCL12-producing cells highly overlap with SCF expressing cells. Conditional ablation of *Cxcl12* from *Lepr*$^{+}$ cells and endothelial cells mobilized HSC and hematopoietic progenitors from the bone marrow to the periphery (Ding and Morrison, 2013b), suggesting that CXCL12/ CXCR4 axis positions and retain HSC and progenitors within the niche. Some overlap may occur among CAR cells, Nestin^{+}, and *Lepr*$^{+}$ cells, which will require further investigation.

Conditional deletion of *Cxcl12* in osteoblasts did not significantly affect the HSC pool but slightly reduced early lymphoid progenitors in BM (Ding and Morrison, 2013b). These results argue that osteoblastic cells regulate lymphoid progenitors. *Lepr*-expressing mesenchymal progenitor cells have been described as the major sources of bone and adipocytes in the marrow (Zhou et al., 2014). Taken together, the data supports a model in which CXCR4/CXCL12 pathway is important for lineage differentiation. Indeed, CXCR4 deficiency during adult hematopoiesis affected predominantly B lymphopoiesis (Ma et al., 1999, Kawabata et al., 1999, Ara et al., 2003a, Nie et al., 2008). The mechanism by which CXCL12 and CXCR4 function to regulate HSC differentiation, namely into the B cell lineage, remains unclear.

5. B Lymphopoiesis

In BM, B lymphocyte development occurs in sequential stages defined by the recombination status of V(D)J genes, cell surface receptors, and also growth factors requirements. Immature B cells egress to the blood to reach secondary lymphoid organs, where they complete their differentiation program (**Figure 1.3**). Antigen-activated mature B cells proliferate and differentiate into plasmablasts, which are fundamental for humoral immune responses, home back and colonize the BM (Manz et al., 1997).

The following sections will focus on the stages of B cell development until their bone marrow egress, and the crucial factors and microenvironments that maintain B cells and their precursors during development in the bone marrow.

5.1 Ontogeny of B cells

In mammals, B cells arise from HSC in the fetal liver (FL) (Gathings et al., 1977, Velardi and Cooper, 1984). Progenitor cells from FL seed the fetal BM, and after birth, BM is the chief site of B lymphopoiesis. In the BM, the interplay between different transcription factors (TF) and specific factors, namely, the cytokines FLT3L and IL-7 instructs a progressive development of B lymphocyte traits while repressing traits of other hematopoietic lineages.

During hematopoietic differentiation, HSC lose their self-renewal potential and transit to a progenitor stage expressing FLT3 denominated MPP (multipotent progenitor), which still maintains the potential to differentiate into all blood cells (Boyer et al., 2011). Gradual expression of recombination of activation genes (*Rag*), terminal deoxynucleotidyltransferase (TdT), and expression of the interleukin-7 receptor (IL-7R) hallmarks the transition to common lymphoid progenitors (CLP). CLP are the first lymphoid-restricted progenitor, and has B, T, natural killer (NK), and dendritic (DC) cell potential (Kondo et al., 1997). Interestingly, CLP can be redirected to the myeloid lineage by stimulation through exogenously expressed IL-2 and GM-CSF (granulocyte/macrophage colony-

stimulating factor) receptors. Moreover, GM-CSF and macrophage colony-stimulating factor (M-CSF) receptors are expressed at low to moderate amounts on HSC, are absent on CLP, and are up-regulated after myeloid induction by IL-2. The data indicates that cytokine signaling instruct lineage differentiation. Plus, the data also supports that a critical step in lymphoid commitment is the down-regulation of cytokine receptors that drive myeloid cell development (Kondo et al., 2000). CLP are a heterogeneous population, which can be further sub-divided by the surface expression of the markers Ly6D and AA4.1 (CD93). Acquisition of Ly6D expression is linked to a higher B lymphocyte lineage potential, suggesting that $Ly6D^+$ CLP is the first B-cell progenitor (Inlay et al., 2009). The development of $AA4.1^+$ CLP is dependent on CXCR4 and CXCL12 (Egawa et al., 2001), but the role of CXCR4 and CXCL12 at the CLP stage is not understood.

The different stages of B cell development have been described by various conventions, which differ in nomenclature (Hardy and Hayakawa, 2001). In the Basel nomenclature, RAG proteins initiate immunoglobulin (Ig) recombination at $cKit^+$ Pro-B cell stage, initiating the transition into Pre-BI cells. At the Pre-BI stage, heavy chain genes are rearranged and surrogate light chain is expressed. The large cycling Pre-BII cells express the pre-BCR, down-regulate cKit, and start expressing IL-2Rα (CD25). At this stage Rag proteins are re-expressed to allow Ig rearrangements. The mature BCR is first expressed on the surface of $CD25^-$ immature B cells (Ernst, 2009).

Hardy's classification distinguishes developmental B cell stages into fractions based on their expression of the surface markers CD43, B220, B.P.1, HSA (CD24), and IgM (**Figure 1.3**). Fractions A (pre-Pro-B cells) to C' (large Pre-B) contain early B cell stages identified as $B220^+CD43^+$ cells and are subdivided by increased expression of HSA and BP.1. Fraction A can be further distinguished by the expression of AA4.1 (CD93); $AA4.1^+$ cells express RAG proteins, are IL-7 responsive, and are thought to represent true B cell precursors (Miller et al., 2002). Mostly after the pre-Pro-B stage, Ig gene rearrangements occur. First, heavy-chain DJ (diversity and junction) occurs, followed by heavy-chain V(variable)DJ rearrangements. Fractions B and C consist of large mitotically active cells, which have heavy-chain DJ rearrangements or VDJ rearrangements, and are named Pro-B cells. Loss of CD43 marks the maturation to small Pre-B (fraction D). Expression of surface

IgM characterizes Immature B cells (fraction E). Mature re-circulating B cells (fraction F) in BM co-express IgD and IgM (Hardy et al., 1991, Nagasawa, 2006).

Immature B cells progress through transitional stages. T1 cells ($IgM^{hi}IgD^{lo}CD93^{+}CD23^{-}$) originate T2 cells, which increase IgD expression and express CD23. T2 cells develop directly into Follicular B cells (FOB) and Marginal Zone B cells (MZ) or become T3 ($IgM^{lo}IgD^{hi}CD23^{+}$) cells. Mature B cells do not express AA4.1, are FOB cells ($IgM^{lo}IgD^{hi}CD21^{int}CD23^{hi}$). In the spleen, non-circulating mature B cells differentiate into MZ cells ($IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo/-}$) (Allman and Pillai, 2008).

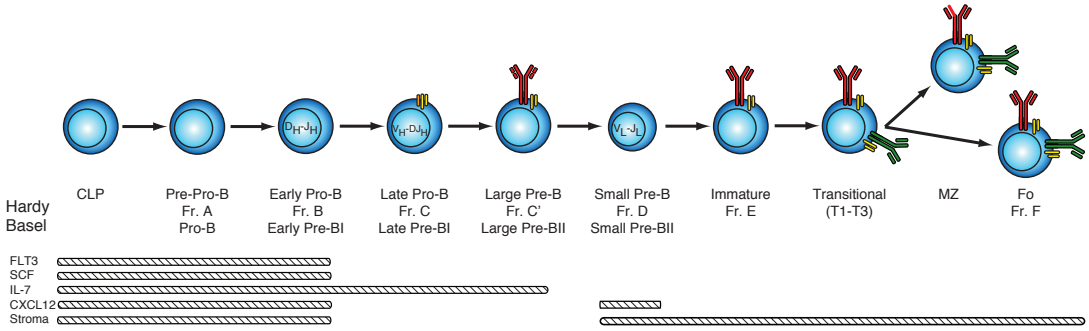

Figure 1.3| Scheme of developmental progression of B lymphoid lineage from CLP to mature B cells in mice. Hatched bars denote growth factor and stroma-dependent stages. Adapted from (Ernst, 2009).

5.2 Essential Factors for B cell development

The functional mechanisms of B cell development have been mostly characterized using in vitro studies. Knockout and transgenic mice have been widely used to understand the mechanisms regulating specific stages of maturation, namely to identify the transcription factors responsible for B cell identity. Transcriptions factors can induce or repress target genes, therefore controlling many events leading to lineage specification, commitment and development. Transcription factors are in turn regulated by growth factors, cytokines, and chemokines, some of which are produced by niche cells. The coordinated crosstalk between the niche environment and cell intrinsic

molecular pathways, such as the transcription factors Ikaros, E2A, and early B-cell factor (EBF) and PAX5, is fundamental for B cell development. Here, I will focus on how chemokines and cytokines instruct and shape B lymphopoiesis.

It has been well established that B cell development in vitro requires a supporting feeding layer of bone marrow-derived stromal cells (Whitlock and Witte, 1982, Whitlock et al., 1987, Collins and Dorshkind, 1987, Hunt et al., 1987), suggesting that in vivo B lymphopoiesis is highly dependent on the interaction between developing B lymphocytes and BM stroma. Therefore, stromal cells most likely attract B cell precursors by means of adhesion molecules and chemokines, such as CXCL12.

CXCR4, the receptor for **CXCL12**, is expressed on subsets of developing B cells and both CXCR4 and CXCL12 are critical for B lymphopoiesis at the earliest stages of development. CXCL12-deficient mouse embryos show severely reduced numbers of the very early B cell precursors ($Lin^-CD19^-cKit^+IL\text{-}7R^+AA4.1^+$ population) in fetal liver. Moreover, B cell precursors are decreased in adult BM in chimeric wild-type mice reconstituted with CXCR4-deficent fetal cells, compared with control mice (Egawa et al., 2001). As mentioned before, CXCR4 has been implied in the retention of early developing B cells in BM (Ma et al., 1999, Nie et al., 2004). However, the number of Pre-B cells in BM of B cell lineage specific conditional CXCR4 knockout is not affected (Nie et al., 2004), suggesting that CXCR4 and CXCL12 act on B cell development at stages before B cell lineage commitment. How CXCR4/CXCL12 regulates B lymphopoeisis at early hematopoietic stages needs to be further investigated.

FLT3L is the ligand for the tyrosine kinase receptor FLT3. FLT3 expression labels all hematopoietic progenitors (Boyer et al., 2011). Deficiency in FLT3 reduces pre-Pro-B, and Pro-B cells numbers by twofold, but Pre-, immature and mature B cells numbers are not significantly affected (Mackarehtschian et al., 1995). By contrast, FLT3L deficiency results in a dramatic reduction in pre-Pro-B cell numbers, slightly reduced Pro- and Pre-B cells numbers, but did not affect more differentiated B lymphocytes (Sitnicka et al., 2002, Sitnicka et al., 2003). The existence of uncharacterized

receptors for FLT3L may explain the phenotypic differences between FLT3 and FLT3L knockout mice (Nagasawa, 2006). Before commitment to B cell lineage, FLT3-deficiency impairs dramatically the CLP population, which also leads to a severe reduction in ETP (emigrant thymocyte progenitors) but only mildly reduces more differentiated thymocytes (Sambandam et al., 2005, Sitnicka et al., 2002). Taken together, these data suggest that FLT3 signaling acts on MPP, CLP, pre-Pro-B cells and ETP. Furthermore, FLT3L is also important for the development of some types of DC (McKenna et al., 2000, Brawand et al., 2002), and NK cells (McKenna et al., 2000), which arise from CLP or pre-Pro-B cells (Rolink et al., 1996, Pelayo et al., 2005, Diao et al., 2004). FLT3L acting synergistically with IL-7 also stimulates pre-pro-B cell growth (Hunte et al., 1996).

The **SCF** and **cKit** pathway is not required for B lymphopoiesis *per se* (Takeda et al., 1997). Instead, SCF is required at the MPP and CLP stages. The cKit-deficiency at CLP stage leads to a block in B lymphopoiesis at the Pro-B stage, in the adult mice (Waskow et al., 2002), when B lymphopoiesis occurs in BM and is highly dependent on IL-7.

RANKL (also known as OPGL and ODF) is a transmembrane protein essential for osteoclast development and for bone remodeling (Kong et al., 1999, Yasuda et al., 1998, Lacey et al., 1998). RANK is the receptor for RANKL, and is expressed by DC, T cells and osteoclast precursors (Anderson et al., 1997, Dougall et al., 1999). RANKL is highly expressed by BM-derived primary stromal cells, osteoblasts and osteocytes (Yasuda et al., 1998, Nakashima et al., 2011). However, RANKL expressed by lymphoid cells is important for B lymphopoiesis (Kong et al., 1999). It is still unknown which RANKL-expressing cells acts on B cell development (Nagasawa, 2006).

IL-7 is essential for adult B lymphopoiesis (von Freeden-Jeffry et al., 1995, Carvalho et al., 2001, Peschon et al., 1994). The stromal compartment in FL, BM, thymus, spleen and lymph nodes produces IL-7. IL-7 binds to IL-7R, which is a heterodimer composed of the IL-7Rα chain and the common γ (γc)

chain. The IL-7Rα chain confers receptor specificity for IL-7 binding (Jiang et al., 2005, Corfe and Paige, 2012). IL-7 binding triggers the heterodimerization of the α and γc chains, resulting in the trans-phosphorylation of the constitutively associated JAK1 and JAK3. Activated JAK kinases phosphorylate tyrosine residues in the α chain. Receptor phosphorylation leads to activation of Src kinases (LYN/ FYN/ Btk) and also creates docking sites for SH2 domain containing proteins PI3K and STAT (Jiang et al., 2005). Phosphorylation of the IL-7Rα chain allows the recruitment of mostly STAT5 and STAT3, which hetero- and homodimerize and then translocate to the nucleus, where they act as transcription factors. STAT3 mediates the survival e differentiation of Pro-, Pre-, and Immature B cells (Chou et al., 2006). **STAT5 carries out the majority of STAT-mediated responses in B lymphopoiesis**. STAT5 is critical for the expression of EBF and PAX5 (Dai et al., 2007). Src kinases potentially help to activate STAT proteins (Ingley and Klinken, 2006).

IL-7/IL-7R signaling is required for the maintenance of the B cell potential at the CLP stage and for survival and proliferation of developing B cells. Deficiency in IL-7 or IL-7R arrests the development of B and T cells but not NK cells or DC (Peschon et al., 1994, von Freeden-Jeffry et al., 1995, Moore et al., 1996, Rodewald et al., 1999). IL-7 deficiency arrest B lymphopoiesis before the Pro-B cell stage (Peschon et al., 1994, von Freeden-Jeffry et al., 1995, Carvalho et al., 2001, Dias et al., 2005). In the periphery, some B lymphocytes are detected when IL-7 is absent. Most likely, these lymphocytes were generated during fetal life, when B cell development is not strictly dependent on IL-7. Fetal derived Pro- and Pre-B cells respond to thymic stromal lymphopoietin (TSLP), whose receptor is formed by IL-7Rα chain and a TSLP-dedicated chain, while in adult BM, only Pre-B cells are TSLP responsive (Vosshenrich et al., 2003, Vosshenrich et al., 2004). Transgenic expression of TSLP in IL-7 knockout mice restored both fetal and adult B cell development, suggesting that TSLP could substitute for IL-7 in fetal B lymphopoiesis (Chappaz et al., 2007). However, deficiency for IL-7 and TSLP did not translate into more severe fetal B lymphopenia. Instead, FLT3L and IL-7 deficiency completely abolished fetal and adult B cell development (Adolfsson et al., 2005, Jensen et al., 2007, Jensen et al., 2008), suggesting that during fetal development FLT3 and IL-7R signaling is critical for B cell

development. Furthermore, FLT3 signaling is fundamental for the residual B cell development in IL-7Rα-deficient mice (Vosshenrich et al., 2003). IL-7 is also fundamental for non-committed hematopoietic progenitors as IL-7 signaling deficiency strongly depletes CLP, specially the $Ly6D^+$ population (Dias et al., 2005, Tsapogas et al., 2011), which leads to a strong arrest in adult BM-dependent B cell development. In addition, CLP isolated from IL-7 deficient mice show reduced ability to generate B cells both in vitro and in vitro (Dias et al., 2005, Tsapogas et al., 2011), and $Ly6D^+$ CLP have lower levels of EBF (Tsapogas et al., 2011). Overexpression of EBF restores the B cell differentiation potential of IL-7 deficient CLP (Dias et al., 2005, Kikuchi et al., 2005). IL-7 signaling also regulates E2A and PAX5 expression (Hirokawa et al., 2003), and promotes V(D)J recombination. IL-7 mediated survival of B cell precursors by inducing the expression of myeloid-cell leukemia sequence 1 (MCL1) protein (Opferman et al., 2003).

After commitment to the B cell lineage, the main function of the IL-7R is to maintain and expand early B cell stages by activating STAT5, inducing in turn the expression of anti-apoptotic factors and cyclin D3, which mediates transit through the cell cycle. Via STAT5, IL-7R signaling leads to hyperacetylation at the distal V-heavy chain segments and thus allows for heavy chain loci accessibility in B cells (Bertolino et al., 2005) and also signals to specifically induce the recombination of D-heavy chain to distal V-heavy chain segments in pro-B cells, by altering the accessibility of DNA substrates to RAG proteins (Corcoran et al., 1998). Complementary signaling through PI3K-AKT pathway represses apoptotic factors and provides the growth and energetic states that are required for proliferation and survival (Clark et al., 2014). In addition, IL-7R signaling also prevents Igλ gene recombination by repressing its locus and by preventing FOXO-induced *Rag* expression (Clark et al., 2014).

Even though IL-7 is fundamental for adult murine B lymphopoiesis, for a long time IL-7 was considered dispensable for human B lymphocyte development. In humans, the disruption of the IL-7R due to genetic mutations causes severe combined immunodeficiency disease (X-SCID), resulting in absence of T lymphocytes and NK cells but no effects on B cell numbers are detected (Puel et al., 1998). Yet, similar to mice, B cells in X-SCID patients may arise during fetal or neonatal development. Co-cultures of

human BM stroma with either cord blood or adult BM HSC were dependent on IL-7 for B lymphopoiesis, while neonatal cord blood generated B lymphocytes independent of IL-7 (Parrish et al., 2009). Similarly to observed in murine models, TSLP does not substitute IL-7 in human B lymphopoiesis, but FLT3L supports IL-7-independent B cell development in neonatal cord blood (Parrish et al., 2009). Human B lymphocyte progenitors express IL-7R and are responsive to IL-7 in a STAT5 mediated manner (Johnson et al., 2005). A recent study using mass cytometry detected phosphorylation of STAT5 in response to IL-7 in a rare population of human B cell progenitors, suggesting that IL-7 plays an instructive role in early B lymphopoiesis in humans (Bendall et al., 2014).

Bone marrow stromal compartment most likely produces and delivers the key factors described above to B cell progenitors. However, the precise identity of the stromal cells supporting B cell development from commitment to differentiation is still not clear.

5.3 B lymphopoiesis supporting cells

Different stromal-cell lines have shown to produce different environmental factors in vitro (Sudo et al., 1989, Zipori et al., 1985b, Zipori et al., 1985c, Zipori et al., 1985a), but whether a single stromal cell type or different types of stromal cells in vivo support B lymphopoiesis is unknown. Recent studies have implicated some BM cell types as the specific cellular niches for B lymphopoieisis.

CXCL12-expressing cells are likely candidate cells to support B lymphopoiesis because CXCL12 is required for the generation of pre-pro-B cells (Egawa et al., 2001). As stated before, different types of BM stromal cells have been described to produce CXCL12 (**table 1.2**). Interestingly, most of CXCL12-producers stromal cells are perivascular and show mesenchymal

stem and progenitor cell characteristics (Omatsu et al., 2010). Therefore, for clarification, these cells will be referred as CAR cells (Sugiyama et al., 2006). CAR cells express VCAM-1 (Tokoyoda et al., 2004), SCF (Ding et al., 2012), Nestin (Mendez-Ferrer et al., 2010b), LEPR (Ding and Morrison, 2013b), platelet-derived growth factor receptor (PDGFRα), CD51 (Pinho et al., 2013), myxovirus resistance-1 (MX-1) (Park et al., 2012), the transcription factor pair related homeobox-1 (PRX-1) that marks cells of the limb bud mesoderm, and the transcription factor Osterix (OSX, also called SP7) (Greenbaum et al., 2013), which is necessary for osteoblast differentiation. Remarkably, mature osteoblast express low amounts of CXCL12 (Greenbaum et al., 2013, Ding and Morrison, 2013b).

Osteoblasts arise from MSC, and are responsible for synthesis, deposition and mineralization of bone extracellular matrix (Nagasawa, 2006, Frenette et al., 2013). Contrary to initial observations (Calvi et al., 2003, Zhang et al., 2003), osteoblasts do not directly regulate HSC maintenance but have been implicated in regulating B cell progenitors (Taichman et al., 1996, Taichman, 2005, Visnjic et al., 2004). Cultures enriched for osteoblasts support B lymphopoiesis (Zhu et al., 2007). In addition, osteoblast depletion affects some B cell progenitors (Visnjic et al., 2004). Conditional deletion of CXCL12 in osteoblasts and their progenitors causes a twofold reduction in CLP (Ding and Morrison, 2013b). Nonetheless, only ~30% of IL-7R^{+}lineage^{-} BM cells localize in proximity to the endosteum (Ding and Morrison, 2013b). Furthermore, CXCL12 expression by osteoblast is low and conditional deletion of CXCL12 in mature osteoblasts (by expression of *Cre* recombinase under the promoter for *Bglap*, which codes for Osteocalcin) did not affect CLP nor B lymphopoiesis (Greenbaum et al., 2013). Consequently, the role of osteoblasts as a niche for B lymphopoiesis needs to be further analyzed. The previous studies accessing a role for osteoblasts as the B lymphoioiesis niche have the caveat of using *Cre* recombinase systems that are active at the progenitor stage. Since osteoblasts arise from MSPC, it is possible that the effects observed are due to the deletion of CXCL12 at the MSPC. It is unknown whether fully differentiated osteoblasts produce IL-7, which is fundamental to support B lymphopoiesis.

IL-7-expressing cells in the BM were first identified by immunohistochemical analysis, and characterized by the expression of VCAM-1, fibroblast-like shape, and lack of CXCL12 expression (Tokoyoda et al., 2004), suggesting that CAR cells and IL-7-expressing cells represent distinct stromal subsets. However, the specific immunostaining for IL-7 is technically challenging (Mazzucchelli et al., 2009). Most likely IL-7 immunostaining only captures a small fraction of the IL-7^{+} cells, perhaps those cells that expressed the highest amount of the cytokine. Therefore, the generation of reporter mice for *Il7* greatly facilitates the detection of IL-7-expressing cells. Using *Il7*$^{GFP/+}$ mice, IL-7 production was detected in VCAM-1^{+} mesenchymal stromal cells, in ~10% of endothelial cells (VCAM-1^{+}CD31^{+}) and in some PDGFα^{+} stromal cells (Hara et al., 2012). Interestingly, data from gene array (Mendez-Ferrer et al., 2010b) and RNA-seq (Kunisaki et al., 2013) studies detected IL-7 and CXCL12 expression in Nestin-expressing MSC. IL-7 expression by a stromal-cell line is induced by stimuli, such as cytokines, and B-cell precursors (Sudo et al., 1989). Consequently, IL-7-expressing cells need to be further characterized in terms of factors expressed, nature and localization within BM parenchyma.

5.4 In vivo model for B lymphopoiesis

Commitment to B cell lineage seems to occur in proximity to the CAR cells as HSC and pre-pro-B cells were shown to be in contact with CAR cells (Tokoyoda et al., 2004, Ding et al., 2012, Ding and Morrison, 2013b, Mendez-Ferrer et al., 2010b, Sugiyama et al., 2006). The observation of B cell precursors in direct contact with stromal cells raises the question of whether adhesion interactions mediate the positioning of developing lymphocytes relative to stromal cells. Cell adhesion is mediated through reversible interactions between cell-surface receptors and their counter-receptors. The observation that BM stromal cells express VCAM-1 (Tokoyoda et al., 2004) suggests that integrins expressed by B cell progenitor cells might have a role in refereeing the adhesion of these cells to stromal cells. Integrins are transmembrane heterodimers of noncovalently paired α and β subunits (Luo

et al., 2007). Integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ belong to the family of leukocyte-specific integrins (Hynes, 2002). α_4 and β_2 integrins mediate leukocyte rolling as well as arrest (Ley et al., 2007). The major ligands for leukocyte integrins are transmembrane glycoproteins with multiple domains that belong to the immunoglobulin superfamily. $\alpha_L\beta_2$ binds to ICAM-1, $\alpha_4\beta_1$ binds to VCAM-1, and $\alpha_4\beta_7$, to VCAM-1 and to mucosal addressin cell adhesion molecule-1 (McEver and Zhu, 2010).

The role of α_4 integrin and its counter parts β_1 and β_7 integrins in the hematopoietic development has been controversial. α_4 integrins have been implicated in hematopoiesis (Williams et al., 1991), lymphocyte homing (Holzmann and Weissman, 1989), and recruitment of leukocytes to the inflammatory focci (Yednock et al., 1992). In addition, $\alpha_4\beta_1$ receptor has been suggested to have a role in the attachment and homing of hematopoietic progenitors to the bone marrow in primates and mice (Papayannopoulou et al., 1995, Papayannopoulou and Nakamoto, 1993, Papayannopoulou et al., 1998). Even though the adult and fetal hematopoietic development are α_4 integrin-independent, the seeding and retention of hematopoietic progenitors in the bone marrow depends on α_4 and β_1 integrins (Bungartz et al., 2006, Potocnik et al., 2000, Brakebusch et al., 2002). Based on these results, α_4 integrin might be mediating the interactions between B cell progenitors and IL-7-producing stromal cells but this integrin would not be critical because the adult B cell development, which is independent on β_1 and β_7 integrins, the counterparts of α_4 integrin (Bungartz et al., 2006). $\alpha_4\beta_1$ integrin has a dominant role in bone marrow homing of hematopoietic progenitors (Papayannopoulou et al., 2001a), but it can synergize with β_2 integrins to retain hematopoietic progenitors in bone marrow parenchyma (Papayannopoulou et al., 2001b). Along these lines, CLPs express $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins and migrate to the thymus (Scimone et al., 2006). These findings indicate that $\alpha_L\beta_2$ integrin is expressed at CLP stage and its activation is dependent on a $G\alpha_i$-dependent pathway.

The current model of B lymphopoiesis implies that CLP are attracted to IL-7 sources in a CXCR4-independent manner, and pre-Pro-B cells relocate to close proximity to CAR cells. Nevertheless, the earliest B cell progenitors are dependent on IL-7 (Dias et al., 2005, Tsapogas et al., 2011), CXCR4 and

CXCL12 (Egawa et al., 2001). It is puzzling how this population is able to receive these critical factors when they are produced by different sources in BM. Moreover, it is also not known the mechanism controlling the migration of the pro-B cells from the CAR cells to the IL-7 expressing cells where they were observed in close proximity (Tokoyoda et al., 2004).

Pre-B cells have been described to be away from both CAR and IL-7-expressing cells and in close contact with Galectin-1 (GAL1) expressing stromal cells (Tokoyoda et al., 2004, Mourcin et al., 2011). GAL1 may be a ligand for pre-BCR (Gauthier et al., 2002) and has been shown to interact with integrins, such as $\alpha_4\beta_1$ on pre-B cells. At the contact area, integrins and their ligands, the pre-BCR and GAL1 form a matrix. Moreover, when the cross-linking of pre-B cells integrins occurs in the presence of GAL1, the pre-BCR is recruited into the synapse, triggering the pre-BCR signaling (Rossi et al., 2006). Most strikingly, GAL1-deficient mice do not show an apparent B-cell phenotype (Espeli et al., 2009). In addition, structural biochemical analyses of the pre-BCR indicate that the pre-BCR cannot bind antigen (Bankovich et al., 2007). Pre-B cells transit between an IL-7-dependent proliferative state to a pre-BCR-dependent stage to subsequently re-express RAG proteins and assemble and express a fully functional BCR (Ochiai et al., 2012, Clark et al., 2014). It is likely that the positioning of pre-B cells relative to IL-7-expressing cells reinforces the switch from a fate that is directed by the IL-7R to one that is determined by the pre-BCR. Downstream of the pre-BCR, IRF4 induces expression of CXCR4 (Johnson et al., 2008), which has been suggested to facilitate the movement of pre-B cells away from IL-7-expressing cells and the attenuation of IL-7R signaling.

Immature B cells are distributed between BM parenchyma and sinusoids, and are particularly dependent on CXCR4 for retention within BM parenchyma (Pereira et al., 2009). However, immature B cells were not seen in contact with CAR or IL-7-expressing cells (Tokoyoda et al., 2004). CXCL12 is a long-range soluble cytokine and thus, immature B cells do not require to be in close proximity to CAR cells. Immature B cells egress from BM parenchyma to the sinusoids. The mechanisms regulating BM B cell egress are not fully understood (see below). Within sinusoids, immature B cells rely on $\alpha_4\beta_1$-VCAM-1 interactions and on the $G\alpha_i$-coupled receptor Cannabinoid receptor 2 (CB2) for their retention. Furthermore, the presence of large

numbers of adherent and migrating immature B cells in the sinusoids suggested that rather than serving solely to support blow flow, these vessels constitute a specialized cellular niche in BM that may influence B cell development. In support of this possibility, CB2 deficiency led to a lower frequency of λ^+ immature B cells in the periphery (Pereira et al., 2009).

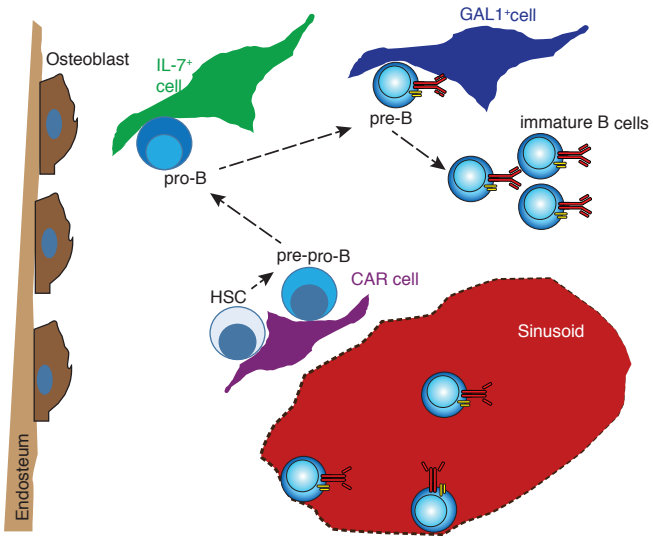

Figure 1.4| Model of B lymphopoiesis in BM. HSC and pre-Pro-B cells are located near perivascular CAR cells (Tokoyoda et al., 2004, Sugiyama et al., 2006), whereas Pro-B cells adjoin IL-7$^+$ cells. Pre-B cells are near GAL1$^+$ cells and away from IL-7$^+$ cells (Mourcin et al., 2011, Tokoyoda et al., 2004). Immature B cells are partitioned between the BM parenchyma and the sinusoids (Pereira et al., 2009).

The regulatory mechanisms of the interactions between developing B cells and their cellular niches remain unclear. It is also still unclear the role of CXCR4/ CXCL12 axis in B lymphopoiesis. It may mediate cell attraction to the niche, enhance developing B cell adherence to the stroma (possibly through activation of integrins), and/or support B cell progenitors survival within the niche (Egawa et al., 2001, Tokoyoda et al., 2004, Nagasawa, 2006).

5.5 Developing B cell egress

Lymphocyte egress from primary and secondary lymphoid organs (SLO) is fundamental for a functional immune system. Lymphocytes continually recirculate by entering SLO from the blood, to then exit SLO into the lymph, and return to circulation via the thoracic duct (Gowans and Knight,

1964, Ford, 1968, Ford, 1969, Cyster, 2005). If egress occurs prematurely during lymphocyte development, it can lead to development of autoimmune disease (Zachariah and Cyster, 2010); if it occurs inefficiently, it can lead to severe lymphopenia and immune deficiency.

Egress occurs when lymphocytes gradually reduce their retention in lymphoid organs while becoming increasingly attracted to exit sites (circulatory fluids, blood and lymph) (Pham et al., 2008). Therefore, egress is dependent on chemokine-mediated migration – chemotaxis, of immune cells. Chemokines bind to their receptor, engaging G-protein coupled receptors (GPCR), predominantly $G\alpha_i$ proteins (Cyster, 2005). Expression of the ADP-ribosylating subunit of pertuxis toxin (PTX) in thymocytes blocked their thymus egress, indicating that $G\alpha_i$-coupled receptors are involved in immune cell egress (Chaffin and Perlmutter, 1991).

$S1PR_1$ is the GPCR responsible for B and T lymphocyte egress from SLO, and T cell egress from the thymus (Cyster and Schwab, 2011). These receptor binds to the bioactive lipid Sphingosine 1-phosphate (S1P). There are five known S1P receptors, named $S1PR_{1-5}$. Naive T and B lymphocytes express high amounts of $S1PR_1$ and lesser amounts of $S1PR_2$, $S1PR_3$, and $S1PR_4$ (Cyster, 2005). S1P is a potent chemoattractant abundant in blood and lymph but less abundant in lymphoid tissue interstitium, creating a concentration gradient, which is fundamental to egress (Schwab et al., 2005, Pappu et al., 2007).

Egress from BM is thought to occur via the vast network of sinusoids. How cells enter the sinusoids is not fully understood. $S1PR_1$ is unlikely to be the only regulator of B cell egress from BM (Pereira et al., 2010b, Allende et al., 2010). Deficiency in S1P/$S1PR_1$ pathway leads to a more than one hundred fold reduction in T and B lymphocytes in the periphery (Matloubian et al., 2004). In contrast, if $S1P_1$ is ablated in developing B cells, only a small decrease of immature B cells is seen in the periphery and a small but statistical significant accumulation of immature B cells in the BM parenchyma (Pereira et al., 2010b, Allende et al., 2010). These observations suggest that $S1PR_1$ may work together with other receptors to promote egress. Other $G\alpha_i$-protein coupled receptors have been implicated in developing B cell positioning in BM. $S1PR_3$ and CB2 retain immature B cells in the sinusoids,

together with the integrin $\alpha_4\beta_1$ and its counterreceptor VCAM-1 (Pereira et al., 2009, Donovan et al., 2010).

CXCR4 expression decreases from pro to pre-B and is further down-regulated in immature B cells, which agrees with the increasing loss of retention. This raises the possibility that $S1PR_1$ signaling overrides the CXCR4-mediated retention, thus promoting B lymphocyte egress. A study has shown that blockade of CXCR4 in $S1PR_1$-deficient B cells did not release immature B cells to the periphery (Allende et al., 2010), supporting this hypothesis. Nevertheless, ablation of CXCR4 mobilizes immature B cells but the majority of pro and pre-B cells remains in the BM parenchyma (Pereira et al., 2009). In addition, overexpression of $S1PR_1$ in pro and pre-B cells does not decrease their number in BM parenchyma (Pereira et al., 2010b). Together these data suggest that additional factors guide immature B cells to egress sites. Ultimately, how immature B cells egress from BM remains poorly understood.

Chapter 2

Aims

Chapter 2 | Aims

Despite their high complexity and variety, immune cells share two characteristics: they originate from a hematopoietic stem cell and they rely on cell migration and positioning to develop and also to carry on immune responses. Cell positioning and migration during immune responses and within secondary lymphoid organs has been broadly studied over the past three decades, but a huge gap lies on the understanding of cell positioning during hematopoiesis.

Except from T lymphocytes, all hematopoietic lineages develop in the bone marrow and are exported to the periphery. The BM stromal and sinusoidal compartment has been implicated in the maintenance of HSC and regulation of their quiescence. However, it is still unknown how hematopoietic progenitors interact and position relative to supporting cells. Hematopoietic lineage differentiation is regulated through the interplay of transcription factors. Besides transcription factors, cytokines have been found fundamental for lineage commitment. In particular, IL-7 is critical in B lymphopoiesis to maintain B lineage cell potential. Strikingly, IL-7 is a secreted cytokine but its levels in serum are very low, suggesting a tight regulation of IL -7 production and a local consumption of the cytokine (Guimond et al., 2009). This suggests that B cell progenitors and developing B lymphocytes have to be positioned in close proximity to IL-7-expressing cells. However, IL-7 producers in bone marrow are poorly characterized. Indeed, only the cellular components responsible for HSC maintenance in bone marrow niche were studied; cellular components of the niche responsible for lineage instruction remain unknown. It is critical to understand whether MPP require signals from HSC niches, or from other BM niches, for lineage differentiation.

CXCR4/CXCL12 is a key organizing chemokine receptor/ chemokine pair in the bone marrow, by controlling fundamental processes of HSC biology, such as quiescence, homing to bone marrow and maintenance in the hematopoietic niche. CXCR4 function on HSC is important for B lymphopoiesis (Nie et al., 2008, Sugiyama et al., 2006), so B lymphocytes are

an ideal immune cell type to understand hematopoietic differentiation niches within bone marrow. We dissociated the role of CXCR4 in HSC from a possible role in hematopoietic progenitors by conditionally deleting CXCR4 in different stages of B lymphopoiesis.

In the present thesis, I sought to address the positional cues responsible for the positioning of hematopoietic progenitors and developing B cells within differentiation niches in bone marrow. Thus, I focused on:

1 – Defining the mechanism of immature B cell egress from the bone marrow (Chapter 3);

2 – Characterizing the lymphoid differentiation niche and the molecular mechanisms regulating the positioning of hematopoietic progenitors within the niche (Chapter 4).

Finally, by comprehending the function of the hematopoietic niches, we aim at solving how extrinsic cues affect lineage output and contribute to leukemogenesis and other hematopoietic disorders.

Chapter 3

CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow

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Chapter 3| CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow

Previous studies had described that CXCR4 and its ligand CXCL12 were critical for B lymphopoiesis (Egawa et al., 2001, Nie et al., 2008). However, deletion of CXCR4 specifically on B lymphocytes did not affect B cell development (Nie et al., 2004). I started my doctoral research by asking whether CXCR4 was required at early stages of B cell development. For that purpose, we conditionally ablated *Cxcr4* expression from the pro-B stage on, using *Mb1*$^{cre/+}$. Fascinatingly, we found that B cell development proceeded normally even though the lack of the retention due to CXCR4-deficiency speeded up their egress from BM. Thus, we looked closer to the role of CXCR4 in the egress of developing B cells. We found that immature B cells down-regulate CXCR4 expression by twofold when they transit from BM parenchyma to the sinusoids. In addition, reduction of CXCR4 expression by using CXCR4 heterozygous immature B cells was sufficient to promote the egress of immature B cells. These data suggested that CXCR4 retention signaling is stronger than the weak egress-promoter signaling of $S1P_1$ in immature B cells (Pereira et al., 2010b). Since egress is mediated by PTX sensitive-Ga_i-coupled receptors (Cyster, 2005), we then proceeded to ask whether other $G\alpha_i$-protein coupled receptor was involved in BM egress of immature B cells. For this purpose, we analyzed mice where all GPCR signaling is exclusively inhibited in developing B lymphocytes by crossing *Rosa26*^{PTX} with *Mb1*$^{cre/+}$ mice. It was surprising that in absence of $G\alpha_i$ function, developing B cells egress faster than wild-type cells, which indicates the existence of $G\alpha_i$-independent mechanism of B cell egress. Simultaneously, in the lab, a fellow graduate student found that immature B cells retention within BM was strictly dependent on amoeboid motility mediated by CXCR4 and CXCL12 and by $\alpha_4\beta_1$ integrin mediated-adhesion to VCAM-1. Moreover, immature B cells near the sinusoids had reduced motility, were predominantly round, and transversed the endothelium in a largely non-amoeboid fashion. The passive mode of cell egress from BM also contributes

significantly to the export of other hematopoietic cells, such as NK cells. Together, our results challenged the paradigm that the BM egress of immune cells is dependent on the S1P/$S1P_1$ pathway, highlighting the egress differences between B and T cells, and were published in the *Journal of Experimental Medicine*. This study also showed that antigen-induced BCR signaling antagonized CXCR4 down-regulation and therefore immature B cell egress from BM.

In this chapter I will present the data I obtained regarding the immature B cell egress project. The full publication can be found in the appendix section.

Abstract

In later stages of B cell development, immature B cells are partitioned between BM parenchyma and the sinusoids, from which they egress to the periphery. In order to egress, lymphocytes gradually reduce their retention in lymphoid organs while become increasingly attracted to exit sites. The bioactive lipid S1P and its receptor $S1P_1$ are strictly required for T cell egress from thymus, and for T and B cell egress from SLOs. Even though immature B cells express abundantly $S1P_1$ and are attracted to S1P gradients in *in vitro* chemotaxis assays, immature B cells still egress with high efficiency when they either lack $S1P_1$, or when S1P is not produced (Pereira et al., 2010b). Here, we show that B cells residing in sinusoids reduced CXCR4 expression by 2-fold, and that a 2-fold reduction is sufficient for promoting B cell displacement from the BM parenchyma, suggesting that CXCR4 is the critical B cell retention chemoattractant in BM parenchyma. Other GPCRs, besides S1P receptors, may contribute to B cells export into the blood. By conditionally expressing PTX in developing B lymphocytes, we found that B-lineage cells were reduced in BM and enriched in peripheral blood and spleen, indicating that cells exited prematurely from BM. These findings show a novel mechanism regulating cell egress from BM that is independent of GPCR signaling. We speculate that $G\alpha_i$-dependent and independent egress mechanisms might occur simultaneously.

1. Introduction

Leukocyte egress from lymphoid organs by active migrating toward exit sites, where they reverse transmigrate across endothelial barriers. Lymphocyte egress from thymus and lymph nodes is critically dependent on the chemoattractant lipid S1P. S1P is abundant in blood and lymph but limited in the lymphoid organs interstitium, creating a sharp concentration gradient. Lymphocytes express the $S1PR_1$, which allows them to sense S1P gradient. Deficiency in $S1PR_1$ reduces by ~50-1,000-fold T and B cell numbers in circulatory fluids, and significantly increases their numbers in lymphoid organs (Cyster and Schwab, 2012). S1PR1 signaling mediates lymphocyte

egress in part by overcoming lymphoid organ retention mechanisms (Pham et al., 2008). BM cell egress juxtaposes to thymus and lymph nodes egress. Genetic or pharmacologically induced S1P receptor deficiency causes an approximately two to three-fold reduction in immature B, NK cells and eosinophil export from BM (Walzer et al., 2007, Jenne et al., 2009, Allende et al., 2010, Pereira et al., 2010b, Sugita et al., 2010), suggesting that S1P and $S1PR_1$ are not the exclusive mediators of BM egress. $S1P_1$ may work together with other receptors to promote immature B lymphocyte egress from BM.

At the immature stage, B cells continue to redistribute away from IL-7^+, and also from GAL1^+ stromal cells, and become competent for export from bone marrow (Mourcin et al., 2011, Tokoyoda et al., 2004). Before exiting BM, immature B lymphocytes are transiently retained within BM sinusoids via a mechanism dependent on $\alpha_4\beta_1$ integrin transactivation by CB2 and adhesion to VCAM-1 (Pereira et al., 2009). Within sinusoidal niches, immature B lymphocytes crawl, detach and re-attach with some cells homing back to the parenchyma while other cells are released into peripheral circulation[175]. $S1RP_3$ has also been implicated in the retention of immature B cells within sinusoids (Donovan et al., 2010).

In late stages of B cell development, immature IgM$^+$ lymphocytes are exported from BM via sinusoids into peripheral blood circulation. S1P/$S1PR_1$ pathway contributes minimally to immature and mature B lymphocyte egress (Pereira et al., 2010a, Allende et al., 2010).

In B cells, CXCR4 is expressed at highest levels in Pro-B cells and, even though its expression decreases as cells progress in B cell development, receptor activity significantly increases in a stepwise manner until the mature B cell stage (Glodek et al., 2003, Pereira et al., 2010b, Pereira et al., 2009). Likewise HSC, B lymphocytes are critically dependent on CXCR4 for homing to, and retention within BM parenchyma (Ara et al., 2003; Broxmeyer et al., 2005; Nagasawa, 2006; Nie et al., 2008; Nie et al., 2004; Peled et al., 1999; Pereira et al., 2009; Sugiyama et al., 2006). CXCL12, the CXCR4 ligand, is a potent chemoattractant to various hematopoietic cells, and is abundantly expressed by non-hematopoietic cells in BM, including stromal cells, osteoblasts, endothelial, and perivascular cells (Sugiyama et al., 2006, Ding and Morrison, 2013a). CXCR4/CXCL12 counteracts the activity of egress-promoting cues in immature B lymphocytes, neutrophils, NK cells and in monocytes (Bernardini et al., 2008, Wang et al., 2009, Allende et al., 2010,

Eash et al., 2010), though how CXCR4 signaling antagonizes cell egress remains unknown.

It is puzzling why the export of T cells from the thymus is solely dependent on S1P/S1PR$_1$ pathway whereas the export of different cells types in BM is not completely dependent on a single GPCR-mediated mechanism. For instance, CXCR2 and CCR2 mediate the BM egress of, respectively, neutrophils and monocytes. Deficiency in either receptor has a small effect in BM egress of these cells (Serbina and Pamer, 2006, Shi et al., 2011, Eash et al., 2010). Two models can be employed to explain BM egress. First, several GPCR play a redundant role in the egress of different cell lineages from BM. Second, alternative mechanisms control hematopoietic cell egress from BM. The second model is supported by the observation that millions of RBC are produced and exported daily from BM (Lichtman and Santillo, 1986), and that these cells lack mechanisms for interstitial amoeboid cell migration.

In this study, we demonstrate that down-regulation of CXCR4 is critical for immature B cell egress from BM. Remarkably, B cell exit from BM was accelerated by lymphocyte intrinsic PTX expression, indicating that the directed migration towards BM exit sites is largely controlled by a passive, GPCR-independent, mechanism. Finally, this passive mode of cell egress from BM is not restricted to developing B lymphocytes but also contributes significantly to NK cell egress from BM.

2. Material and Methods

Mice. Adult C57BL/6 (CD45.2$^+$), Boy/J (CD45.1$^+$) were obtained from The Jackson Laboratories. *Cxcr4*$^{Fl/Fl}$, *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ transgenic mice were from internal colony. *Cxcr4*$^{Fl/-}$ and *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ mice were crossed with *Mb1*$^{cre/+}$ mice (Pelanda et al., 2002) to generate *Cxcr4*$^{Fl/-}$ *Mb1*$^{cre/+}$ and *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Mb1*$^{cre/+}$ mice. *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ mice were crossed to *Il7ra*$^{cre/+}$ to generate *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Il7ra*$^{cre/+}$, and control littermates. *Il7ra*$^{cre/+}$ mice were provided by Dr. Hans-Reimer Rodewald (German Cancer Research Center). Mice were maintained under specific pathogen-free conditions at Yale Animal Resources

Center, and used according to the protocol approved by the Yale University Institutional Animal Care and Use Committee.

Tissue preparation, cell enumeration, and antibodies. BM cells were flushed from femurs and tibias in DMEM (Cellgro) containing 2% FBS (Invitrogen), 5% of antibiotics (Cellgro), and Hepes (Cellgro). PB was collected from the portal vein, and erythrocytes were lysed with NH4Cl, KHCO3, and EDTA. Spleen was dissociated in 5 ml DMEM containing 2% FBS, 5% of antibiotics, and Hepes using a cell strainer (Thermo Fisher Scientific). Cells were counted with a Coulter Counter (Beckman Coulter). B cells were identified by staining with anti-B220 (RA3-6B2) or CD19 (1D3), anti-IgM (11/41), anti-IgD (11-26c.2a), and anti-CD93 (AA4.1) antibodies. NK cells were stained with CD3e (145-2C11) and NK1.1 (PK136) antibodies. Dead cells were excluded by staining with DAPI. Cells were analyzed by FACS (LSRII; BD).

In vivo labeling of BM sinusoidal B cell subsets. BM sinusoidal cells were labeled by injecting i.v. 0.3 µg phycoerythrin-conjugated rat anti-mouse CD19 in 200 µl PBS. 2 min after, mice were sacrificed in a CO_2 chamber.

BM chimeras. Approximately $1.5x10^6$ total BM cells from $Ly5.1^+$ donors were mixed with $1.5x10^6$ total BM cells from adult Boy/J ($Ly5.2^+$) mice and were transferred into adult Boy/J mice that had been exposed to two rounds of 6.35 Gy separated by 3 h. Chimeras were analyzed at least 6 wk after reconstitution.

Transwell migration assays. Chemotaxis assays were performed using 10^6 BM or spleen cells incubated for 30 min with 1x DMEM containing 0.5% fatty acid–free BSA (EMD Biosciences), 5% of antibiotics, L-glutamine (Cellgro), and Hepes. Cells were then allowed to migrate through 5-µm-pore–sized transwells (Corning) toward soluble CXCL12 (R&D Systems), 2-AG (Cayman), or CXCL13 (PeproTech) for 3 h at 37C. Cells were collected, stained, and

resuspended in 40 μl of staining buffer and analyzed by flow cytometry for 40 s.

Statistics. Student's two-tailed *t* test was performed using GraphPad Software Prism 6 or Microsoft Excel. A value <0.05 was considered significant.

3. Results

CXCR4 is essential for immature B cell retention in BM

To identify at which stage B developing cell require CXCR4 the most, we conditionally ablated CXCR4 expression in developing B cells by crossing *Cxcr4*$^{Fl/-}$ mice with *Mb1*$^{cre/+}$ mice. We found that Pro and Pre-B cell subsets were not affected in BM but immature B cells were significantly mobilized into the peripheral blood (**Fig. 3.1a** and **b**). CXCR4 is intrinsically required for immature B cell egress as CXCR4-deficient immature B cells are mobilized from BM parenchyma into sinusoids when in competition with CXCR4-suficient cells (**Fig. 3.1c**). The data indicates that after commitment to B cell lineage, CXCR4 is mostly required to retain immature B cells in the BM parenchyma.

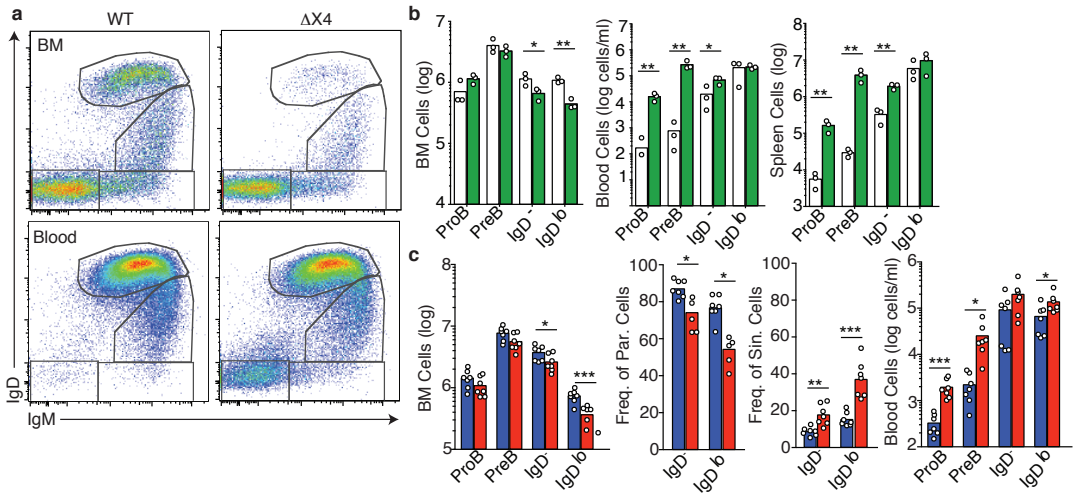

Figure 3.1| CXCR4 is intrinsically required in B lymphocytes for retention in bone marrow. **a,** Flow cytometic analysis of IgM and IgD expression in live-gated B220^{+} cells in BM and blood of *Mb1$^{Cre/+}$;Cxcr4$^{+/+}$* (WT) and *Mb1$^{Cre/+}$;Cxcr4$^{Fl/-}$* (ΔX4) mice. **b,** Enumeration of B-lineage cells in BM, blood and spleen of *Mb1$^{Cre/+}$;Cxcr4$^{+/+}$* (white) and *Mb1$^{Cre/+}$;Cxcr4$^{Fl/-}$* (green) mice. **c,** Frequency of Ly5.1^{+} developing B cell in BM (first panel), immature B cell subsets in parenchyma and sinusoids (middle panels), and developing B cells in blood (last panel). Data is representative of three independent experiments. Bars indicate average, and circles indicate individual mice. * P<0.05; ** P<0.005; by unpaired Student's *t* test.

Immature B cell egress is independent of chemotaxis mediated by Gα_i-protein coupled receptor signaling

Lymphocyte egress from lymphoid organs is controlled by a *tug of war* between tissue retention signals and chemoattractants emanating from, or associated with, endothelial hot spot exit sites (Cyster and Schwab, 2012). As chemoattractants are sensed predominantly by PTX-sensitive GPCRs, we asked whether immature B lymphocyte egress from BM requires intrinsic GPCR signaling. To address this question, we conditionally induced PTX expression in B-lineage cells by introducing *Mb1$^{Cre/+}$* alleles into mice carrying the ADP-ribosyl transferase S1 subunit of PTX within the *Rosa26* locus preceded by a premature transcriptional stop codon flanked by loxP sites (Regard et al., 2007).

The number of Pro and Pre-B cells was slightly but significantly reduced in BM, which corresponded to a small, but highly significant

numerical increase in blood circulation (**Fig. 3.2a** and **b**). Remarkably, IgM^+ immature B cell subsets were reduced by 2-3 fold in BM and significantly increased in blood, indicating increased BM egress. In order to evaluate a possible leakiness of PTX expressed by developing B cells into the BM interstitium, we carefully analyzed the behavior of wild-type cells (Ly5.2) developing in the same environment as congenically marked wild-type or PTX-expressing B cells (Ly5.1). As Pro and Pre-B cells are rare in the peripheral blood of wild-type animals, but are readily detected if expressing PTX (**Fig. 3.2c**; upper panels show Ly5.1 gated cells), we reasoned that significant PTX leakage would enforce Pro and Pre-B cell mobilization from BM irrespectively of the genotype. As seen in panel **d** (lower panels show wild-type LY5.2 gated cells), the frequency of Pro and Pre-B cells in blood of WT/WT or PTX/WT mixed chimeras is indistinguishable. The frequency of Ly5.1/Ly5.2 reconstitution was similar between both sets of mixed chimeras (Fig. 3.2d). The frequency of Ly5.1+ PTX-expressing (orange) Pre-B cells in blood is significantly increased as compared to $Ly5.1^+$ wild-type (black) Pre-B cells due to inefficient retention in BM, as expected (**Fig. 3.2e**). In contrast, the frequency of wild-type $Ly5.2^+$ Pre-B cells in peripheral blood of mixed chimeras repopulated with PTX-expressing B-lineage cells is similar to that found in blood of mixed chimeras reconstituted with wild-type cells (**Fig. 3.2f**). From these data we conclude that PTX is not leaked to the BM interstitium in significant amounts. Furthermore, the faster egress of PTX-expressing developing B cells were caused by $G\alpha_i$-protein inhibition in B-lineage cells as similar findings were obtained with B-lineage cells deficient in *Gnai2* and *Gnai3* (Hwang et al., 2013). PTX-expressing B-lineage cells were also poorly retained within BM sinusoids (**Fig. 3.2g** and **h**), consistent with a role for $G\alpha i$-coupled CB2 and S1PR3 in this process (Donovan et al., 2010, Pereira et al., 2009). PTX-expressing B cells progressed normally throughout development (**Fig. 3.2i**) and showed largely similar kinetics of BrdU incorporation during B cell development as wild type cells (**Fig. 3.2j**).

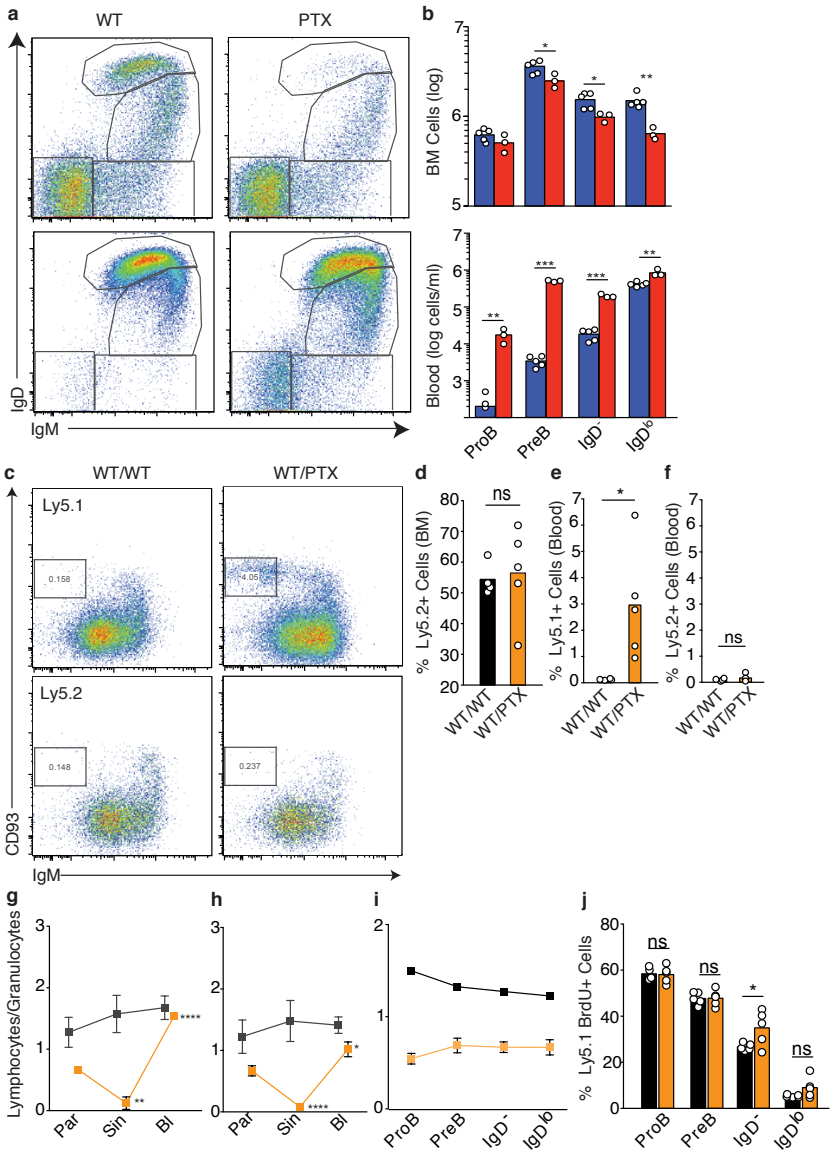

Figure 3.2| B cells egress from bone marrow independently of GPCR-mediated migration. **a,** Flow cytometry analyses of IgM and IgD expression in live gated (DAPI-) B220+ cells in BM (upper panels) and peripheral blood (lower panels) of *Mb1*$^{Cre/+}$ mice (WT, left panels) and *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice. **b,** Quantification of developing B cell subsets in BM (left) and peripheral blood (right) of *Mb1*$^{Cre/+}$ mice (blue bars) and *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice (red bars). Bars indicate mean, and circles depict individual mice analyzed. Data is representative of more than 3 independent experiments. **c-j,** Effects of intrinsic PTX expression in B cell development, and lack of evidence for PTX leakage into neighboring cells. **c,** Flow cytometric analyses of peripheral blood taken from lethally irradiated mice reconstituted with 50% of either Ly5.1$^+$ *Mb1*$^{Cre/+}$ *Rosa26*$^{+/+}$ or Ly5.1$^+$ *Mb1*$^{Cre/+}$ *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ BM cells and 50% of congenic Ly5.2$^+$ WT cells. Left and right panels show WT/WT and WT/PTX mixed chimeras gated on B220$^+$ Ly5.1$^+$ cells (top) or on B220$^+$ Ly5.2$^+$ cells (bottom), respectively. **d,** Frequency of Ly5.2$^+$ cells within the Pre-B cell gate in BM of WT/WT (black) and WT/PTX (orange) mixed chimeras. Note that both experimental groups had similar BM chimerism. **e,** Frequency of Ly5.1$^+$ Pre-B cells within the B220$^+$ gate in peripheral blood of WT/WT (black) and WT/PTX (orange) mixed

chimeras. Note that PTX-expressing Ly5.1$^+$ Pre-B cells are significantly increased in blood. **f,** Frequency of Ly5.2$^+$ Pre-B cells within the B220$^+$ gate in peripheral blood of WT/WT (black) and WT/PTX (orange) mixed chimeras. Bars indicate average; circles indicate individual mice analyzed. **g,** Distribution of immature CD93$^+$ IgM$^+$ IgD$^-$ cells in BM parenchyma, sinusoids and peripheral blood of WT/WT (black) and WT/PTX (orange) mixed chimeras. **h,** Distribution of immature CD93$^+$ IgM$^+$ IgD^{lo} cells in BM parenchyma, sinusoids and peripheral blood of WT/WT (black) and WT/PTX (orange) mixed chimeras. **i,** Chimerism of developing B cell subsets in WT/WT (black) and WT/PTX (orange) mixed chimeras. Y-axes display the frequency of Ly5.1$^+$ cells in Pro-B cells divided by the frequency of Ly5.1$^+$ cells within the granulocyte gate. Cells within BM parenchyma and sinusoids were distinguished by intravenous administration of PE-conjugated anti-CD19 antibodies 2 minutes before euthanasia. Lines represent average; and error bars indicate SEM of 5 individual mice analyzed in each group. **j,** Frequency of BrdU$^+$ cells within Ly5.1$^+$ developing B cell subsets of WT/WT (black) and WT/PTX (orange) mixed chimeras. Bars indicate average; circles represent individual mice analyzed (n=5 in each group). Data in all panels are representative of two independent experiments. ns, not significant; * P<0.05, ** P<0.005, **** P<0.00005 by unpaired Student's *t* test.

We also observed that AMD3100 blocked the chemotaxis of wild type and PTX-expressing developing B cell subsets towards a CXCL12 gradient in vitro (**Fig. 3.3a and b**), whereas CXCR4-deficient B cells were unable to migrate in similar conditions (**Fig. 3.3c-e**). These observations suggest that CXCR4 can couple to PTX-insensitive G proteins in B cells, which in turn may also be involved in CXCR4-mediated retention in vivo.

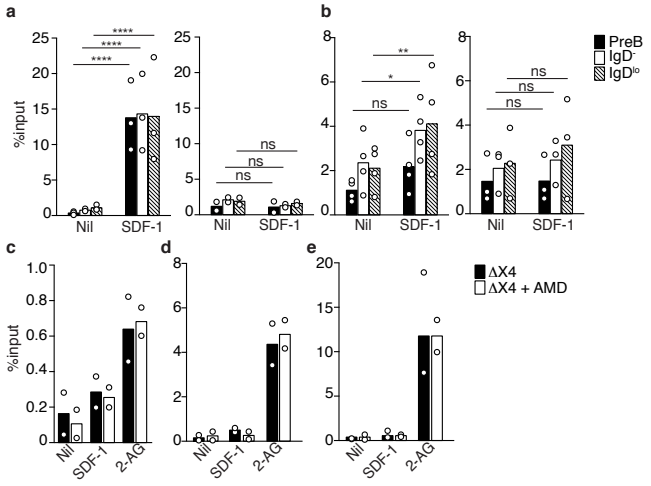

Figure 3.3| CXCR4 can promote B-lineage cell migration through a PTX-insensitive mechanism. a, In vitro transwell migration assay of untreated WT BM B cell subsets towards 0.3mg/mL of CXCL12 untreated (left panel) or treated with the CXCR4 antagonist AMD3100 (right panel). **b**, In vitro transwell migration assay towards 0.3mg/mL of CXCL12 of PTX-

expressing BM B cell subsets untreated (left panel) or treated with the CXCR4 antagonist AMD3100 (right panel). **c-e,** In vitro transwell migration assay of CXCR4-deficient Pre-B (**c**), immature IgD^{-} (**d**), and immature IgD^{lo} (**e**) B cell subsets untreated (black) or treated with AMD3100 (white) towards 0.3mg/mL of CXCL12 (SDF-1), or towards 1mM 2-arachidonoyl glycerol (2-AG). Bars indicate average; circles indicate independent experiments. ns, not significant. * P<0.05; ** P<0.005; *** P<0.0005; **** P<0.00005 by unpaired Student's *t* test.

The GPCR-independent egress mechanism also mediates NK cells egress from BM

Next, we asked whether NK cells could also leave BM independently of PTX-sensitive GPCRs. To address this question, we expressed PTX in NK cells by *Cre*-mediated recombinase driven by *Il7ra* (Schlenner et al., 2010), and quantified the egress of NK cells from BM into peripheral blood by flow cytometry. Remarkably, NK cells, like B-lineage cells, were efficiently mobilized from BM into blood by PTX treatment (**Fig. 3.4**), even though PTX-sensitive GPCRs can contribute to their export from BM (Eash et al., 2010, Allende et al., 2010, Jenne et al., 2009, Serbina and Pamer, 2006, Walzer et al., 2007, Pereira et al., 2010a).

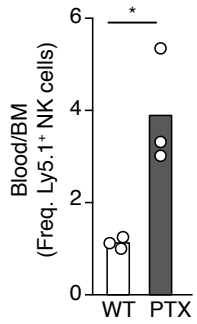

Figure 3.4| PTX mobilizes NK cells from BM into peripheral blood. Analysis of NK cells in BM and peripheral blood of lethally irradiated mice reconstituted with a mixture of bone marrow cells isolated from *Ly5.1*$^{+/+}$ *Il7ra*$^{cre/+}$ *Rosa26*$^{+/+}$ (white), or from *Il7ra*$^{cre/+}$ *Rosa26*$^{PTX/+}$ mice (blue), with *Ly5.2*$^{+/+}$ mice. NK cells were gated as CD19^{-} CD3e^{-} NK1.1^{+}. Y axis shows the ratio of the frequencies of Ly5.1^{+} NK cells in blood and BM. Bars indicate the mean; circles depict individual mice. Data was pooled from 3 independent experiments. * P<0.05; ** P<0.005; *** P<0.0005; and **** P<0.00005, by unpaired Student's *t* test.

Gradual reduction in CXCR4 expression enforces immature B cell egress from BM

CXCR4 expression is highest at the Pro-B cell stage and decreases gradually during murine and human B cell development (Honczarenko et al., 1999, Pereira et al., 2009). Therefore, it is possible that CXCR4 signaling is reduced in egress-competent cells. To investigate this possibility we compared CXCR4 expression on immature B lymphocyte subsets before and after BM egress, and found that it was reduced by approximately 2-fold in cells within sinusoids as compared to cells in parenchyma (**Fig. 3.5a and b**). To test if a 2-fold down-regulation of CXCR4 was sufficient to mobilize immature B cells from BM we compared the distribution of CXCR4 haploinsufficient B-lineage cells in competition with wild type cells in BM mixed chimeras. As expected, *Cxcr4*$^{+/-}$ immature B cells in BM parenchyma expressed comparable CXCR4 surface amounts as *Cxcr4*$^{+/+}$ B cells positioned within BM sinusoids, whereas *Cxcr4*$^{+/-}$ immature B cells expressed ~ 2-fold lower CXCR4 than *Cxcr4*$^{+/+}$ immature B cells in BM parenchyma (**Fig. 3.5c**). Furthermore, *Cxcr4*$^{+/-}$ immature B cells were significantly reduced in BM parenchyma, and increased in BM sinusoids and in blood when compared to *Cxcr4*$^{+/+}$ immature B cells (**Fig. 3.5d**).

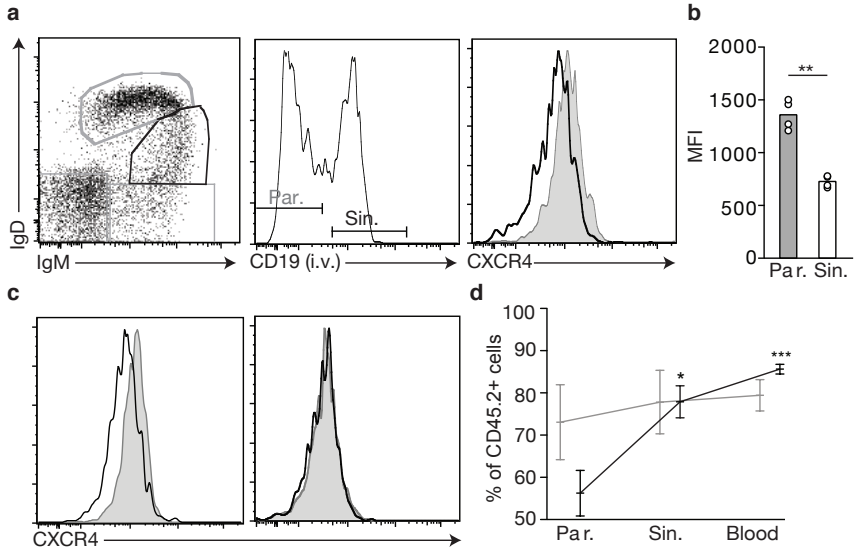

Figure 3.5| CXCR4 expression in egress-competent immature B lymphocytes. a, CXCR4 expression in B220^{+} immature IgM^{+} IgD^{lo} B cells positioned in BM parenchyma (Par.) and sinusoids (Sin.). (left) IgM and IgD expression in B220^{+} BM cells. (middle) Distribution of B220^{+} CD93^{+} IgM^{+} IgD^{lo} B cells in BM parenchyma (CD19-PE^{-}) and sinusoids (CD19-PE^{+}). (right) CXCR4

expression in B220^{+} immature IgM^{+} IgD^{lo} B cells in parenchyma (gray) and sinusoids (black line). **b,** Geometric mean fluorescence intensity (MFI) of CXCR4 surface expression in BM parenchyma and sinusoid immature CD93^{+} IgM^{+} IgD^{lo} B cells from C57BL/6 mice (n = 4). Bars indicate the mean; circles indicate individual mice. Data are representative of >10 independent experiments. **c**, (left) CXCR4 surface expression in *Mb1*$^{Cre/+}$;*Cxcr4*$^{Fl/+}$ immature IgM^{+} IgD^{lo} cells overlaid with *Mb1*$^{Cre/+}$;*Cxcr4*$^{+/+}$ immature IgM^{+} IgD^{lo} in BM parenchyma. (right) CXCR4 surface expression in Ly5.1^{+} *Mb1*$^{Cre/+}$;*Cxcr4*$^{Fl/+}$ immature IgM^{+} IgD^{lo} in BM parenchyma overlaid with Ly5.2^{+} (*Cxcr4*$^{+/+}$) immature IgM^{+} IgD^{lo} cells residing in sinusoids. **d**, Distribution of B220^{+} CD93^{+} IgM^{+} IgD^{lo} immature B cells in BM parenchyma, sinusoids, and PB of lethally irradiated mice reconstituted with a mixture of Ly5.1^{+} *Mb1*$^{Cre/+}$;*Cxcr4*$^{+l/+}$ and Ly5.2^{+} WT cells (gray) or with Ly5.1^{+} *Mb1*$^{Cre/+}$;*Cxcr4*$^{Fl/+}$ and Ly5.2^{+} WT cells (black). Error bars indicate ±SEM. Bars indicate mean; circles indicate individual mice. Data were pooled from three independent experiments. *, P < 0.05; **, P < 0.005; ***, P < 0.0005 by unpaired Student's t test.

4. Discussion

During B cell development, progression into the immature B cell stage is characterized by a significant change in positional distribution within BM. About 50% of immature B cells localize in BM sinusoids in a $\alpha_4\beta_1$/VCAM-1, and CB2 and S1PR$_3$ signaling-dependent manner (Donovan et al., 2010, Pereira et al., 2009). Immature B cell movement towards sinusoids was independent of CB2 and S1PR$_3$-mediated chemotaxis, and only minimally contributed by S1P and S1PR$_1$ (Allende et al., 2010, Pereira et al., 2010a). Our studies reveal that CXCR4 down-regulation, which occurs gradually during mouse and human B cell development (Honczarenko et al., 1999, Pereira et al., 2009), was critical for immature B cell egress from BM. Rather than depending on the developmental expression of single or multiple egress-promoting GPCR, immature B lymphocytes egress BM by down-regulating CXCR4 by approximately 2-fold.

It is surprising that in absence of Gα_i function, developing B cells egress faster than wild-type cells, which indicates the existence of a Gα_i-independent mechanism of B cell egress. Erythrocyte egress from BM has been shown to be driven by pressure drop across the sinusoid wall.

Interestingly, the authors referred to it as *vis-a-tergo* from the Latin, a force from behind (Lichtman and Santillo, 1986). In agreement with these studies, it has been recently demonstrated using intravital imaging that megakaryocytes extend proplatelet-containing membrane protrusions into the sinusoidal compartment and these intravascular extensions appeared to be sheared from their transendothelial stems by flowing blood, resulting in the appearance of proplatelets in peripheral blood (Junt et al., 2007). These results highlight the possibility that blood flow–induced hydrodynamic shear stress is a biophysical determinant of BM egress. Therefore, we hypothesize that $G\alpha_i$-independent mechanism of BM B cell egress might involve shear stress. Recent studies from our laboratory demonstrated that circulatory molecules rapidly perfuse BM parenchyma (Beck et al., 2014). Thus, it is possible that BM parenchyma has a high blood flow-induced hydrodynamic shear stress, which is sufficient to mobilize the cells out of BM in absence of $G\alpha_i$-signaling. BM egress occurs through the vast sinusoidal network. In order to the cells reach the sinusoids, they have to transmigrate through the endothelium lining the sinusoidal compartment. Using TEM imaging, it has been observed that megakaryocytes and erythrocytes enter the lumen of the vascular sinus through the parajunctional zones of the endothelial cell. Although a passive role of the endothelium cannot be excluded, the observation of a radial and circumferential microfilaments associated with apertures in endothelial cell cytoplasm suggest an active participation of endothelial cells in the reverse transmigration of leukocytes from BM parenchyma to the sinus lumen. In addition, migration of cells occasionally occurs through thick areas of endothelial cytoplasm and the pores are formed at the time of migration and sealed when migration is complete. The authors also speculate that the formation of the aperture might also be related to specific interactions between the leukocyte and the endothelium (Chamberlain and Lichtman, 1978). Recent evidence has shown that neutrophils have a reverse transendothelial migration (Woodfin et al., 2011). Therefore, another possible $G\alpha_i$-independent mechanisms might be specific lymphocyte-endothelium interactions that modulate the endothelium permeability.

The BM parenchyma is considerably perfused by an extensive network of sinusoids formed by a thin-walled endothelium (Tavassoli and Yoffey,

1983). Some studies have demonstrated that most sinusoids are permeable to large molecules due to fenestrations between adjacent endothelial cell processes, and allow exchange of parenchymal interstitium and sinusoidal plasma (Tavassoli and Yoffey, 1983, Mazo et al., 1998). The interstitial fluid flow generates considerable shear force and, under these conditions, developing B lymphocytes may be pushed to exit sites, and leave the BM in a GPCR-independent mechanism.

GPCR-independent egress mechanism also operates on NK cells, suggesting that this mechanism operates in the export of the different hematopoietic cells from the BM. For instance, inflammatory monocytes rapidly egress the BM (Shi et al., 2011, Serbina and Pamer, 2006); but RBC leave the BM carried by interstitial fluid flow in a passive manner into the blood (Lichtman and Santillo, 1986). Considering that all blood cells produced in BM enter circulation via the sinusoids (Tavassoli and Yoffey, 1983), we propose that the BM microanatomy evolved unique strategies for exporting highly heterogeneous cell populations. The egress of non-motile and of slow moving cells is largely independent of egress-promoting chemoattractants near, or at, exit sites, and predominantly occurs via passive transport by interstitial fluid drainage into collecting sinusoids. Large collecting venules receive blood from a network of smaller and irregular sinusoids linked by short anastomoses, and blood flows unidirectionally in these large venules (Mazo et al., 1998). In contrast, smaller sinusoids often show unpredictable fluctuations in the direction of blood flow (Mazo et al., 1998), and it is possible that interstitial fluid drainage from parenchyma into small sinusoids contributes to changes in flow direction. Whether smaller sinusoids with irregular blood flow constitute BM egress sites remains unclear.

Chapter 4

HSC niches control multipotent progenitor differentiation

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The results described in this chapter are in revision at *Immunity*.

Chapter 4| Hematopoietic stem cell niches control multipotent progenitor differentiation

In the previous chapter, we demonstrated that CXCR4-deficiency does not compromise the later stages of B cell development, even though later stage developing B cells are prematurely exported into the periphery, suggesting that CXCR4 plays a role in B lymphopoiesis before lineage commitment occurs. Therefore, we ablated CXCR4 function at different early stages of hematopoiesis and found that CXCR4 is critical for the $Ly6D^+$ CLP, a B lineage primed progenitor population. This phenotype resembled the phenotype observed in IL-7 or IL-7R deficiency. Thus, we determined the positioning of $Ly6D^+$ CLP relative to IL-7 cells using reporter mice, and found that $Ly6D^+$ CLP are positioned in close proximity to IL-7-expressing cells. Moreover, these progenitors rely on CXCR4 to consume IL-7, as CXCR4-deficiency correlates with lower levels of STAT5 phosphorylation.

We also described that IL-7-expressing cells form a subset of CAR cells. Furthermore, we also ruled out osteoblast and the endosteum as a niche for B lymphopoiesis. Collectively, our findings provide groundbreaking evidence supporting a model in which HSC maintenance and multilineage differentiation are distinct cell lineages decisions locally controlled by HSC niche. Our results were submitted and are in revision at *Immunity*.

Abstract

Hematopoietic stem cells (HSC) self-renew in bone marrow niches formed by CXCL12^{+} mesenchymal progenitor and endothelial cells. Here, we show that hematopoietic multipotent progenitors (MPPs) encounter lineage-instructive differentiation signals in HSC niches. Conditional deletion of CXCR4 in MPPs profoundly reduced differentiation into common lymphoid progenitors (CLPs), which significantly decreased lymphopoiesis. CXCR4 was required for CLP positioning near IL-7^{+} cells and, consequently, for optimal IL-7R signaling. IL-7^{+} cells form a large subset of CXCL12-abundant reticular mesenchymal progenitor cells capable of differentiation into osteoblasts and adipocytes, and a minor subset of BM sinusoidal endothelial cells. Conditional *Il7* deletion in mesenchymal progenitor cells dramatically reduced B-lineage committed CLPs, whereas *Il7* deletion from endothelial cells specifically reduced preB cells. Our studies demonstrate that CLPs rely on positional cues to encounter short-range lymphopoietic signals provided by CXCL12^{+} mesenchymal progenitors. Thus, HSC maintenance and multilineage differentiation are distinct cell lineage decisions controlled by HSC niches.

1. Introduction

In mammals, HSCs are maintained throughout life in specialized niches in bone marrow (BM). Upon cell division, HSCs either self-renew to maintain the HSC pool, or differentiate into diverse hematopoietic cell subsets. Over the past few years several studies have identified rare mesenchymal stem and progenitor cells as essential cellular components of HSC niches in BM that control HSC quiescence and long-term maintenance (Morrison and Scadden, 2014). However, the existence of a HSC differentiation niche remains unclear.

CXCR4 and its ligand CXCL12 form a chemokine/chemokine receptor pair that controls multiple essential fetal and adult hematopoietic processes.

Early studies using mice genetically deficient in CXCR4 or CXCL12 demonstrated a severe reduction in B lymphopoiesis and a mild reduction in myelopoiesis in the fetal liver, and severe impairment in myeloid, lymphoid, and megakaryocyte cell development in fetal BM (E18.5) (Ma et al., 1998, Nagasawa et al., 1996, Zou et al., 1998). Some of these defects were in part explained by defective retention of hematopoietic precursors in BM, and by other findings indicating that CXCR4 is also required for hematopoietic stem cell homing and retention in BM (Ara et al., 2003b, Lapidot and Kollet, 2002, Ma et al., 1999, Peled et al., 1999). Furthermore, CXCR4 signaling in HSC was proposed to be critically required for HSC quiescence (Nie et al., 2008; Sugiyama et al., 2006).

CXCL12 is expressed abundantly by mesenchymal progenitor cells, named CXCL12-abundant reticular (CAR) cells (Omatsu et al., 2010; Sugiyama et al., 2006). CAR cells are essential organizers of HSC niches in BM because these cells regulate HSC quiescence and long-term maintenance (Ding and Morrison, 2013; Greenbaum et al., 2013; Omatsu et al., 2014), at least in part through the production of short-range signals such as stem cell factor (SCF) (Ding et al., 2012). Thus, a model emerges in which CXCR4 enables HSCs to position near CAR cells in order to access critical factors controlling HSC decisions in BM. In favor of such a model, HSC have been found in proximity to Nestin-expressing mesenchymal stem and progenitor cells (MSPC) that also express CXCL12 (Kunisaki et al., 2013; Mendez-Ferrer et al., 2010). Indeed, MSPC share many morphological and functional similarities with CAR cells, including multipotent progenitor differentiation potential and expression of high amounts of SCF (Omatsu et al., 2010), suggesting a significant overlap between these cell types.

Amongst the many hematopoietic lineages, B lymphocytes are the most critically dependent on CXCR4 and CXCL12 (Nie et al., 2008; Sugiyama et al., 2006). This dependence is likely to be at an early hematopoietic stage given the fact that conditional deletion of CXCR4 in Pro-B cells did not impair B cell development in BM (Beck et al., 2014; Nie et al., 2008; Pereira et al., 2009). These findings led us to ask the question of how could a single chemoattractant receptor (CXCR4) control HSC quiescence and lymphopoiesis. One possibility is that defects in HSC quiescence directly cause hematopoietic differentiation defects regardless of signals provided by

BM niches. Alternatively, CXCR4 and CXCL12 guide HSC and hematopoietic multipotent progenitors (MPP) to BM niches that not only support HSC quiescence but also sustain MPP differentiation.

B lymphopoiesis is dependent on interleukin -7 (IL-7; Carvalho et al., 2001; von Freeden-Jeffry et al., 1995). IL-7 signals through a heterodimeric receptor composed by an IL-7Ra chain and a common g-chain (Corfe and Paige, 2012). IL-7 binding to IL-7Rα leads to phosphorylation and activation of Janus kinase 1 and 3 (JAK1 and JAK3), in addition to IL-7Rα phosphorylation, which serve as docking sites for the recruitment of signal transducer and activator of transcription 5a and 5b (STAT5) (Corfe and Paige, 2012). Bound STAT5 is phosphorylated by JAK1 and JAK3, which results in its dissociation from IL-7Rα, and translocation to the nucleus where it activates gene transcription (Corfe and Paige, 2012). Despite these studies, very little is understood about the identity of IL-7-producing cells in BM.

Here in show that CXCR4 was intrinsically required in MPPs for differentiation into multiple downstream lineage-restricted hematopoietic precursors, most prominently to the earliest B cell-committed common lymphoid precursor (CLP) that expresses Ly6D (Inlay et al., 2009). We show that CXCR4 controlled the positioning of Ly6D$^+$ CLPs in the vicinity of IL-7$^+$ cells in BM, and that defective positioning near IL-7$^+$ resulted in reduced IL-7R signaling in Ly6D$^+$ CLPs. We further show that IL-7 is primarily made by a subset of CAR cells, which also contains multipotent mesenchymal differentiation capacity. Our studies demonstrate that positional cues play critical roles in enabling hematopoietic precursors to access short-range lineage-instructive cytokines. Furthermore, our studies favor a model in which HSC quiescence and multilineage differentiation are locally controlled by HSC niches in the BM.

2. Materials and Methods

Mice. Adult C57BL/6 (CD45.2$^+$), Boy/J (CD45.1$^+$), *Lepr*-cre, Rosa26$^{FloxSTOP\text{-}tdTomato/+}$, *Tie2*-cre, *Prx1*-cre, and *Rosa26*$^{YFP/+}$ mice were purchased from The Jackson Laboratories. *Cxcr4*$^{Fl/Fl}$ and *Il7*$^{Fl/Fl}$ mice were from internal colony. *Il7ra*$^{cre/+}$ mice were kindly provided by Dr. Hans-Reimer Rodewald (German Cancer Research Center). *Flk2*-cre mice were a gift from Dr. E. Camilla Forsberg (University of California, Santa Cruz). *Il7*-ECFP mice were provided by Dr. Scott Durum (National Institute of Health). Some *Il7*$^{GFP/+}$ mice were from our internal colony (Kyoto University); some *Il7*$^{GFP/+}$ mice were obtained from Dr. Susan Kaech (Yale University). *Cxcl12*^{DsRed} were a kind gift from Dr. Sean Morrison (University of Texas Southwestern Medical Center). *Il7*-cre mice were received from Dr. Ellen Richie (The University of Texas MD Anderson Cancer Center). *Col2.3*-cre mice were kindly provided by T. Nakamura (Kyoto University). *Vav*-cre mice were kindly provided by Dr. Thomas Graf (Albert Einstein College of Medicine). All mice were maintained under specific pathogen-free conditions at Yale Animal Resources Center and in the Experimental Research Center for Infectious Diseases in the Institute for Virus Research, Kyoto University, and used according to the protocol approved by the Yale University Institutional Animal Care and Use Committee and by the Animal Research Committee at the Institute for Virus Research, Kyoto University.

Bone marrow chimeras. Adult Boy/J (CD45.1) mice were exposed to two rounds of 6.35 Gy separated by 3h using an XRAD 320 X-ray irradiator (Precision X-ray Inc.). A mixture of CD45.2$^+$ and CD45.1$^+$ bone marrow cells were injected iv. Recipient mice were sacrificed 6 weeks after transplantation and bone marrow, blood, spleen, Peyer's Patches, lymph nodes (inguinal, mesenteric, auxillary and brachial), thymus, liver and peritoneal cavity were harvested to analyze donor chimerism of Granulocytes, B, T and NK cells.

Flow cytometry. Bone marrow cells were isolated by flushing long bones with DMEM (Lonza) containing 2% heat-inactivated fetal bovine serum (FBS),

HEPES, PenStrep and L-Glutamine (Cellgro). Red blood cells were lysed with ammonium chloride buffer. Spleen, lymph nodes, Peyer's Patch, and thymus were mashed and cells were dissociated to single cell suspension and then filtered through a 70 μm nylon mesh. Liver leukocytes were isolated using Percoll gradients. For flow cytometric analysis of stromal cells, bone marrow was carefully flushed using HBSS supplemented with 2% of heat-inactivated fetal bovine serum. Whole bone marrow was digested with 200 U/mL Collagenase IV (Worthington Biochemical Corporation), and digested at 37ºC for 20 min. Cells clumps were dissociated by gentle pippeting, followed by a further incubation of 10 min at 37ºC. Cells were washed with 5 mL of HBSS/2%FBS, spun at 1000 rpm for 7 min, and stained with the respective antibodies. All stains were done for 20 min on ice, followed by 2 washes at 1000 rpm, 7 min. For detection of pSTAT5a, bone marrow cells were flushed from long bones in DMEM containing 0.5% fatty acid free BSA (EMD Biosciences), antibiotics (50 IU/mL of ampicillin and 50 mg/mL of streptomycin), and 10 mM HEPES. Cells were fixed in 2% PFA for 10 min at RT and permeabilized with 90% Methanol for 30 min and washed with PBS. pSTAT5a was detected using Alexa Fluor 647-conjugated antipSTAT5a antibody (BD Pharmigen). Additional antibodies used are listed in table below.

Table 7.1| List of antibodies and fluorochromes used, and vendors.

Antibody	Clone	Conjugated	Company
anti-goat IgG		Alexa Fluor 647	Jackson Immunoresearch
anti-rat IgG		Alexa Fluor 647	Jackson Immunoresearch
anti-rabbit IgG		AMCA	Jackson Immunoresearch
anti-rabbit IgG		Biotin	Jackson Immunoresearch
CD3e	145-2C11	FITC	Biolegend
CD3e	145-2C11	Pe-Cy7	Biolegend
CD3e	145-2C11	APC	Biolegend
CD3e	145-2C11	Biotin	BD Biosciences
CD4	RM4-5	FITC	Biolegend

CD4	GK1.5	Pe-Cy7	Biolegend
CD8a	53-6.7	Pe-Cy7	Biolegend
CD11c	N418	FITC	Biolegend
CD11c	N418	Biotin	Biolegend
CD11b	M1/70	FITC	Biolegend
CD11b	RM2815	Biotin	Invitrogen
CD16/32	93	PE	Biolegend
CD19	1D3	FITC	Biolegend/ BD Biosciences
CD19	6D5	Pac. Blue	Biolegend
CD19	1D3	PerCP-Cy5.5	eBioscience
CD19	MB19-1	Biotin	eBioscience
CD25	eBio74	Biotin	eBioscience
CD31	390	APC	Biolegend
CD34	RAM34	Alexa Fluor 700	BD Biosciences
CD41	MWReg30	FITC	Biolegend
CD44	IM7	PerCP-Cy5.5	Biolegend
CD45R (B220)	RA3-6B2	PerCP-Cy5.5	eBioscience
CD45.1	A20	FITC	Biolegend
CD45.1	A20	PE	BD Biosciences
CD45.1	A20	Pe-Cy7	eBioscience
CD45.1	A20	Pac. Blue	Biolegend
CD45.2	104	FITC	BD Biosciences
CD45.2	104	PE	eBioscience
CD45.2	104	Pac. Blue	Biolegend
CD45.2	104	APC-Cy7	Biolegend
CD45.2	104	Alexa Fluor 700	Biolegend
CD93 (AA4.1)	AA4.1	APC	eBioscience

CD117 (cKIT)	2B8	Biotin	Biolegend
CD117 (cKIT)	2B8	APC-H7	BD Biosciences
CD117 (cKIT)	2B8	APC	BD Biosciences
CD117 (cKIT)	2B8	BV421	Biolegend
CD127	A7R34	PE	Biolegend
CD127	A7R34	eFlour450	eBioscience
CD127	A7R34	Purified	BioXCell
CD135 (FLT3)	A2F10	PE	eBioscience
CD144	BV13	Alexa Fluor 647	Biolegend
CD150	mShad150	PerCP-eF710	eBioscience
CD184 (CXCR4)	2B11	PerCP-eF710	eBioscience
CD184 (CXCR4)		Biotin	BD Biosciences
GFP		purified	Invitrogen
Gr1	RB6-8C5	FITC	Biolegend
Gr1	RB6-8C5	Biotin	eBioscience
IgD	11-26c.2a	PE	Biolegend
IgM	II/41	Pe-Cy7	eBioscience
LepR		Biotin	R&D
Ly6A/E (Sca-1)	D7	Pe-Cy7	eBioscience
Ly6D	49-H4	FITC/ PE	Biolegend
NK1.1	PK136	FITC	Biolegend
NK1.1	PK136	PerCP-Cy5.5	BD Biosciences
NK1.1	PK136	Biotin	eBioscience
Osteocalcin	mOC(1-20)	purified	Clonotech
Osteopontin		purified	R&D
Perilipin	D1D8	purified	Cell Signaling
pSTAT5	47/STAT5(pY694)	Alexa Fluor 647	BD Biosciences

Streptavidin		Alexa Fluor 488	Invitrogen
Streptavidin		PE	BD Biosciences
Streptavidin		PerCP-Cy5.5	BD Biosciences
Streptavidin		Alexa Fluor 647	Invitrogen
Streptavidin		BV 605	Biolegend
Streptavidin		Qdot605	Invitrogen
Ter-119	Ter119	FITC	Biolegend
Ter-119	Ter119	Biotin	BD Biosciences

Mesenchymal progenitor cell sorting. Long bones recovered from 3-4 C57BL/6 mice (ages 6-12 week) were flushed and digested with Collagenase as described above. Cells were counted and stained with anti-LEPR antibody for 1 hour, on ice. LEPR staining was revealed with biotin-conjugated anti-goat for 40 min. After washing, cells were incubated with anti-CD45, anti-Ter119, anti-CD31, anti-CD144 antibodies, Streptavidin BV605, and DAPI for 30 min. Cells were acquired and sorted on a BD FACS Aria II equipped with UV (355nm), Violet (405nm), Blue (488nm), Green (532nm) and Red (640nm) lasers, and with FACSDiva 7.

Immunostaining of bone marrow sections. Freshly dissected long bones were fixed in a paraformaldehyde-based fixative at 4ºC for 6h or overnight. Then, bones were washed in PBS 1x, for 10 min, 3 times. Bones were dehydrated in a solution of 30% sucrose in PBS, at 4ºC overnight. Samples were embedded in OCT and snap-frozen in ethanol/dry ice bath. For wholemount staining, bones were shaved on a cryostat until the bone marrow cavity was fully exposed. Frozen sections were prepared according to the Kawamoto method (Kawamoto, 2003) or using CryoJane tape transfer system (Leica). 7-μm or 25-μm sections were dried overnight. Sections were rehydrated in PBS with 0.05% BSA for 10 min before staining. Goat serum (4%) in PBS with 1%BSA was used for blocking. Primary antibodies were

applied to the slides for 3h at room temperature or overnight at 4ºC, and this was followed by secondary antibody incubation by 1-2h at room temperature with repeated washes in between. Sections were incubated with a blocking solution of rat serum (4%), BSA (1%) in PBS at least for 30 min. Then, fluorochrome-conjugated antibodies were applied to the slides for at least 1h. Slides were mounted with Fluormount-G (SourthernBiotech) or with a 30% glycerin solution. Images were acquired on a Zeiss Z1 Observer fluorescent microscope equipped with Colibri LED light sources, or on a Leica SP8 confocal microscope.

Chemotaxis assay. Total bone marrow cells were stained against lineage markers, and incubated with Dynabeads (Sigma). A Lin- cell enriched cell suspension was recovered and incubated for 30 min at 37ºC in DMEM containing 0.5% fatty acid free BSA (EMD Biosciences), antibiotics (50IU/mL of ampicillin and 50 mg/mL of streptomycin; Cellgro), and 10 mM HEPES (Cellgro). Cells were allowed to migrate through 5 μm-pore sized transwells (Corning) for 3h at 37ºC with 5% CO2. Cells were collected, stained, resuspended in 45μL of staining buffer and analyzed by flow cytometry for 40s.

CLP single cell differentiation in vitro assays. OP9 stromal cells were seeded in 96-well plates (2,500 cells/well), left overnight at 37 ºC, and irradiated (10 Gy) before plating of hematopoietic cells. Single live Ly6D$^+$ CLP (Lin$^-$ CD127$^+$ cKIT^{int} SCA1$^+$ FLT3$^+$ Ly6D$^+$) were sorted to each well. RPMI (containing 10%FBS, HEPES, PenStrep, L-Glutamine, and 50μM β-Mercaptoethanol) supplemented with saturating amounts of Flt3L, SCF, IL-7 and IL-2 (final concentration of 50 ng/mL) was added to each well. On day 5, culture medium was replaced with RPMI supplemented with FLT3L and IL-7only. On day 10 of culture, scores were assigned for B cells (CD19$^+$), myeloid (CD11b$^+$), NK cells (NK1.1$^+$) and dendritic cells (CD11c$^+$) by flow cytometry.

Adipocyte isolation. Bone marrow from long bones was gently flushed with HBSS/2% FBS supplemented with 200U/mL Collagenase IV and DNase I (Roche), and incubated at 37ºC for 15 min. After digestion, the cell suspension was washed and allowed to stand for 10 min, and then spun at 500g for 5 min. Adipocyte layers were gently recovered from the upper phase.

In vitro osteoblast differentiation. Neonatal calvaria osteoblasts were prepared from 3-5 day old C57BL/6 mice. Briefly, calvariae were treated with 4mM EDTA in PBS and sequentially digested with collagenase (200 U/mL) for 5 times. Fractions 3-5 were plated in α-MEM containing 10% FBS, 10 mM HEPES, and Penn/Strep. Media was changed every 3-4 days. Adult osteoblasts were differentiated as follows: BM cells flushed from femurs and the bones were incubated in DMEM containing 2% Collagenase A (Roche) and antibiotics at 37˚C with agitation for 30 min (500 µl/bone), then washed with DMEM. Bones were incubated a second time in DMEM containing 2% Collagenase A and antibiotics at 37˚C with agitation for 30 min and this fraction was collected. To remove contaminating hemaetopoietic cells, cells were incubated with biotin anti-CD45 (Clone 30-F11, Biolegend) at 1:100 dilution in 100 µl of DMEM containing 2% FBS for 20 min at 4˚C then washed two times, cells were then resuspended in 100 µl of DMEM containing 10 µL strepavidin beads (Invitrogen) and incubated at 4˚C with gentle shaking for 45 min. Labeled hemaetopoietic cells were separated using a magnetic field and remaining cells were plated up to $1x10^6$ per well on 6 well plates in α-MEM containing 10% FBS, 10 mM HEPES, 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 10 nM glycerophosphate (Sigma), and 10 nM dexamethasone (Sigma). Media was changed every 3-4 days.

RNA isolation and Quantitative Real Time PCR. Total RNA was isolated from sorted stromal cells, adipocytes and cultured osteoblasts using mRNeasy kit (Qiagen). cDNA was synthetized from the isolated RNA and Q-PCR was the SensiFASTTM SYBR Lo-ROX Kit (Bioline) and the CFX TouchTM Real-Time PCR detection system (BioRad). *Hprt* mRNA levels were used control. Murine PCR primer sequences: *Bglap*-Fw, GGG CAA TAA GGT AGT

GAA CAG; *Bglap*-Rv, GCA GCA CAG GTC CTA AAT AGT; *Adipoq*-Fw, TGT TCC TCT TAA TCC TGC CCA; *Adipoq*-Rv, CCA ACC TGC ACA AGT TCC CTT; *Plin1*-Fw, CAT CTC TAC CCG CCT TCG AA; *Plin1*-Rv, TGC TTG CAA TGG GCA CAC T; *Il7*-Fw, GCT GCC TGT CAC ATC ATC TG; or GCC ACA TTA AAG ACA AAG AAG GT (for gene deletion); *Il7*-Rv, CAG CAC GAT TTA GAA AAG CAG C or TGG TTC ATT ATT CGG GCA AT (for gene deletion); *Cxcl12*-Fw, CGC CAA GGT CGT CGC CG; *Cxcl12*-Rv, TTG GCT CTG GCG ATGT GGC; *Hprt* -Fw, AGG TTG CAA GCT TGC TGG T; *Hprt* -Rv, TGA AGT ACT GAT TAT AGT CAA GGG CA.

3. Results

CXCR4 controls CLP development in BM

In order to dissociate the role played by CXCR4 in HSC quiescence from its role in MPP differentiation, we conditionally deleted *Cxcr4* exclusively in hematopoietic MPPs using a Flk2-cre transgene (Boyer et al., 2011). To further restrict multilineage differentiation from adult BM niches we reconstituted lethally irradiated, congenically marked, wild-type (WT) mice (CD45.1$^+$ C57BL/6) with a mixture of BM cells from *Cxcr4*$^{Fl/Fl}$;*Flk2*-cre$^+$ or *Flk2*-cre$^-$ mice (both CD45.2$^+$) with BM cells from WT mice (CD45.1$^+$ C57BL/6), and analyzed hematopoietic cell lineages in BM and in the periphery six weeks after reconstitution. The frequency of hematopoietic cell subsets that differentiated from *Cxcr4*$^{Fl/Fl}$;*Flk2*-cre$^-$ (WT, CD45.2$^+$) or from competitor precursors (WT, CD45.1$^+$) was similar to engrafted cells (not shown). In contrast, CXCR4-deficient MPP were reduced by 2-fold, multipotent CLP were reduced by 3-fold, B-lineage restricted Ly6D$^+$ CLPs (Inlay et al., 2009) were reduced by more than 8-fold, and developing B cell subsets were nearly absent from BM (**Fig. 4.1A**, and for gating strategy see Figure S1). Using this approach CXCR4 deletion was detectable at the MPP and CLP stages, and was evident at the Pre-B cell stage (Figure S2A). CXCR4-deficient megakaryocyte and erythroid progenitors (MEP), and granulocyte and monocyte progenitors

(GMP) were also significantly reduced by 2-fold in BM (**Fig. 4.1B**). The 3-fold reduction in multipotent CLPs led to a similar reduction in early thymic precursors (ETP; **Fig. 4.1C**). We also detected reduced numbers of lymphoid precursors and lymphocyte subsets in non-irradiated *Cxcr4*$^{Fl/Fl}$;*Flk2*-cre^{+} and *Cxcr4*$^{Fl/Fl}$;*Flk2*-cre^{-} adult mice, albeit the fold change was less prominent than that seen in mixed BM chimeras (Figure S2B and C). Although hematopoietic progenitors, including CLP and early B cell subsets, were mobilized to the periphery (Figure S2D) as expected (Nie et al., 2008), it nevertheless resulted in a profound reduction in total T, B and NK cell production (**Fig. 4.1D**). Because CXCR4 was required for MPP differentiation into CLP subsets (**Fig. 4.1A**), the profound defect in lymphoid lineage production could have been caused by defective CLP differentiation. To address this possibility we conditionally deleted *Cxcr4* from CLP using *Il7ra*$^{Cre/+}$ mice (Figure S2A, E and F) and performed similar BM mixed chimeras. Although multipotent (Ly6D^{-}) CLP were normally represented in BM (**Fig. 4.1E**), ETP were significantly reduced (**Fig. 4.1F**), and B-lineage committed Ly6D^{+} CLPs were significantly reduced by 3-fold in BM (**Fig. 4.1E**). These defects also resulted in a significant 2-3 fold reduction in total T, B and NK cells produced (**Fig. 4.1G**), despite the mobilization of lymphoid precursors into the spleen (Figure S2F). These data showed that CXCR4 was essential at the MPP and CLP stages for their retention in BM, and for the generation of a full lymphocyte compartment. These data also revealed a requirement for CXCR4 in MPP for their efficient differentiation into other hematopoietic cell lineage-restricted progenitors (e.g. MEP and GMP).

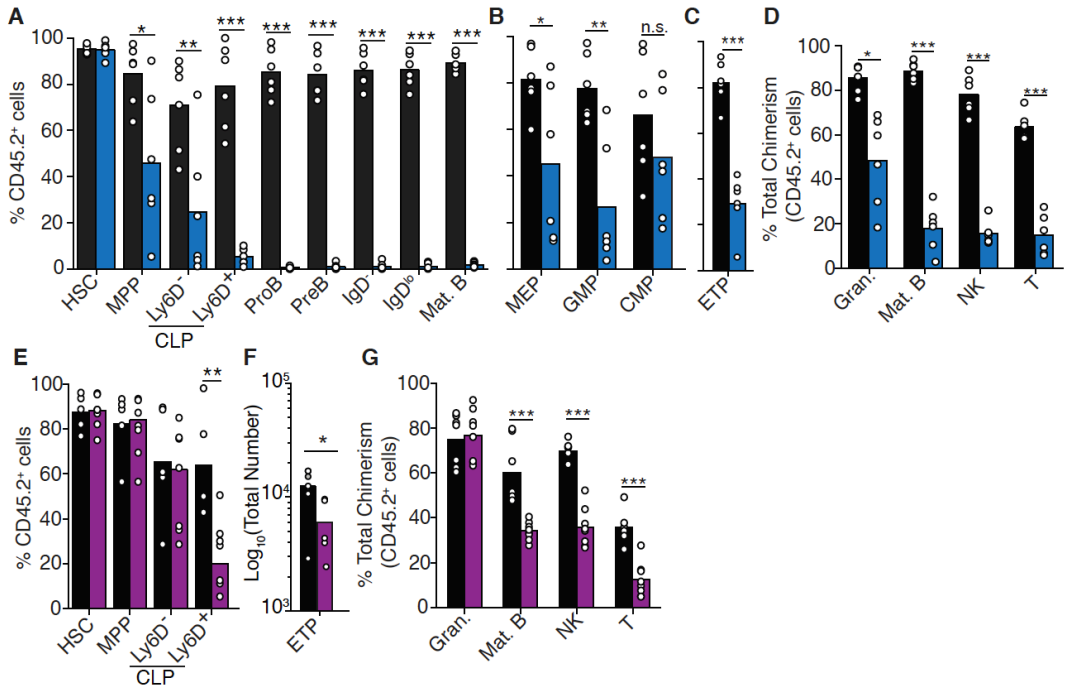


Figure 4.1| CXCR4 intrinsic expression in MPPs and CLPs is critical for lymphoid cell development. (A-D) Frequency of CD45.2$^+$ hematopoietic cell subsets in BM (A and B), thymus (C), and in all lymphoid organs (D) of mice reconstituted with 90% CD45.2$^+$ *Flk2*-cre$^-$;*Cxcr4*$^{Fl/Fl}$ (black) or *Flk2*-cre$^+$;*Cxcr4*$^{Fl/Fl}$ (blue) cells mixed with 10% CD45.1$^+$ WT cells (data pooled from three independent experiments). (E) Frequency of CD45.2$^+$ HSC, MPP and CLP subsets in BM of mice reconstituted with 90% CD45.2$^+$ *Il7ra*$^{Cre/+}$;*Cxcr4*$^{Fl/Fl}$ (purple) or *Il7ra*$^{Cre/+}$;*Cxcr4*$^{+/+}$ (black) cells and 10% WT CD45.1$^+$ cells. (F) ETP number in *Il7ra*$^{Cre/+}$;*Cxcr4*$^{Fl/Fl}$ (purple) and control mice (black). (G) Frequency of CD45.2$^+$ in hematopoietic cells taken from all lymphoid organs (total chimerism). Data pooled from 3 independent experiments. Bars indicate the average; circles depict individual mice. * P<0.05, **P<0.01, ***P<0.001 (unpaired, two-tailed Student's *t*-test).

CXCR4 guides hematopoietic precursors towards IL-7$^+$ cells in BM

Next, we asked how CXCR4 controls lymphoid lineage differentiation. The developmental block at the MPP to CLP transition, and particularly to the Ly6D$^+$ CLP stage, was reminiscent of the phenotype of *Il7* deficient mice (Carvalho et al., 2001; Dias et al., 2005; Tsapogas et al., 2011). This similarity led us to hypothesize that CXCR4 was required at the CLP stage for optimal IL-7/IL-7R signaling. To test this hypothesis we analyzed the distribution of Ly6D$^+$ CLPs relative to IL-7-expressing cells. Even though IL-7

has been detected by immunofluorescence microscopy in situ (Tokoyoda et al., 2004, Tokoyoda et al., 2009), its visualization is difficult and may be restricted to cells that express IL-7 at highest levels. Thus, we visualized Ly6D$^+$ CLPs in femur BM of *Il7*-ECFP transgenic mice (Mazzucchelli et al., 2009), and of *Il7*$^{GFP/+}$ knock-in mice (Miller et al., 2013). In both *Il7* reporter mice, IL-7$^+$ cells could only be visualized after signal amplification with anti-GFP antibodies (data not shown), which strongly suggests that *Il7* is expressed at very low amounts in vivo. B-lineage committed CLPs were identified in situ as being negative for hematopoietic cell lineage markers (see Materials and Methods for details), IL-7Rα^+, and Ly6D$^+$ (**Fig. 4.2A**), a cell gate that is ~93% enriched in Ly6D$^+$ CLP (Figure S3A and B). Similar CLP frequencies were detected by immunofluorescence microscopy and by flow cytometry (Figure S3C and D). We found that more than 90% of Ly6D$^+$ CLP were positioned within <15 μm from IL-7$^+$ cells, whereas IgD$^+$ B lymphocytes were predominantly distal to IL-7$^+$ cells (**Fig. 4.2B** and **C**). Interestingly, Ly6D$^+$ CLP proximity to IL-7$^+$ cells was dependent on intrinsic CXCR4 expression (**Fig. 4.2D** and **E**) and, thus, their positioning was sensitive to a 3-day treatment with the CXCR4 antagonist AMD3100 (**Fig. 4.2F** and **G**). Defective CLP positioning near IL-7$^+$ stromal niches resulted in a significant reduction in IL-7Rα signaling, as measured by STAT5a phosphorylation (**Fig. 4.2H** and **I**).

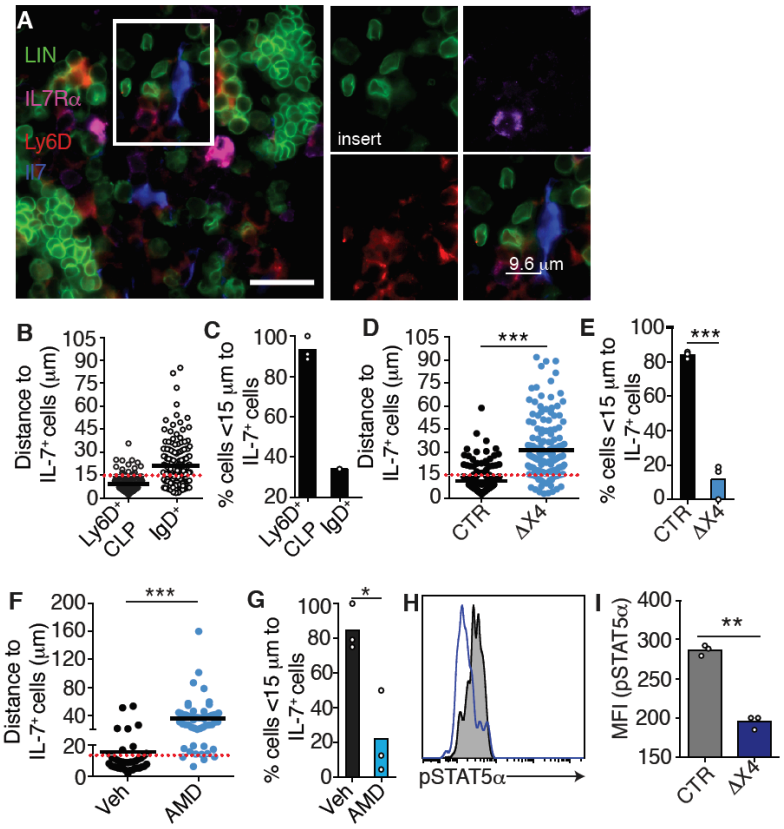


Figure 4.2| CXCR4 controls CLP positioning near IL-7$^+$ cells in BM. (A) 7 μm-thick section of *Il7*-ECFP transgenic femur. Lin. (green) IL-7Rα (pink), Ly6D (red) and *Il7* (blue). Scale bar, 20 μm. (B) Ly6D$^+$ CLP (n=166), IgD$^+$ (n=164), and IL-7$^+$ cell distribution in BM. (C) Ly6D$^+$ CLP and IgD$^+$ cell frequency near IL-7$^+$ cells (<15 μm). (D and E) distribution of *Flk2*-cre$^+$;*Cxcr4*$^{Fl/Fl}$ (blue, ΔX4) or *Flk2*-cre$^-$;*Cxcr4*$^{Fl/Fl}$ (black, CTR) Ly6D$^+$ CLPs, and IL-7$^+$ cells (D); cell frequency near IL-7$^+$ cells (<15 μm) (E). (F and G) Distribution of Ly6D$^+$ CLPs and IL-7$^+$ cells after AMD3100 (AMD) or vehicle (Veh) treatment for 3 days (F). Cell frequency near IL-7$^+$ cells (<15 μm) (G). (H and I) pSTAT5a in *Il7ra*$^{Cre/+}$;*Cxcr4*$^{Fl/+}$ (CTR) and *Il7ra*$^{Cre/+}$;*Cxcr4*$^{Fl/Fl}$ (ΔX4) Ly6D$^+$ CLPs. (H). pSTAT5a geometric mean intensity (I). Bars indicate mean; circles depict individual mice. Data pooled from 3 independent experiments. * P<0.05, **P<0.01, ***P<0.001 (unpaired, two-tailed Student's *t*-test).

CXCR4 expression and function is increased at the CLP stage when compared to earlier HSC and MPP stages (**Fig. 4.3A** and **B**). As CXCR4 signaling has been suggested to control HSC quiescence (in addition to its role as a chemoattractant receptor) we asked whether CXCR4 signaling was also required for CLP differentiation into B-lineage cells. To test this possibility, we measured the efficiency of CXCR4 sufficient and deficient single cell-sorted CLPs to differentiate into B and NK cells in vitro. We found

that CXCR4 sufficient and deficient CLP differentiated similarly into B and NK cells when cultured on OP-9 stromal cell layers with exogenous IL-7 (**Fig. 4.3C**). As OP-9 cells express CXCL12 (**Fig. 4.3D**), the ability of CXCR4 sufficient and deficient CLPs to differentiate equally into B and NK cells suggests that CXCR4 signaling is not required for lymphoid cell development from CLP *per se*. Combined, these data suggest that CXCR4 controls lymphopoiesis by promoting CLP positioning near IL-7^{+} cells in BM, which enables IL-7Rα signaling and STAT5a phosphorylation.

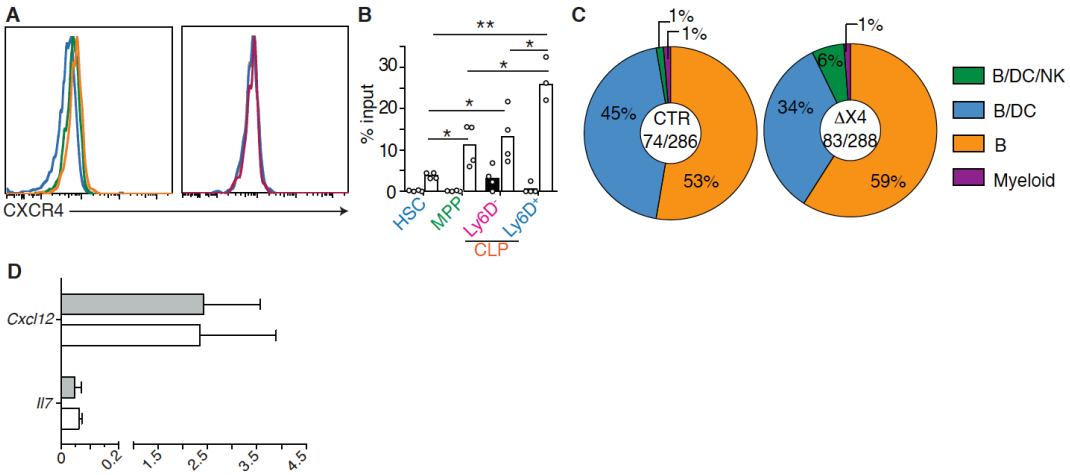


Figure 4.3| CXCR4 expression and function in HSCs and hematopoietic progenitor cell subsets. (A) CXCR4 surface expression in HSC (blue), MPP (green), total CLP (orange, left), and in Ly6D^{-} (pink) and Ly6D^{+} (cyan) CLP (right). (B) In vitro cell chemotaxis through 5 μm transwells: nil (black), CXCL12 (white; 300 ng/mL). Bars indicate mean, circles depict individual experiments. (C) In vitro single cell CLP differentiation on OP-9 stromal cells for 12 days in the presence of IL-7 (50 ng/mL). (D) *Il7* and *Cxcl12* mRNA expression in non-irradiated (white) and irradiated (gray) OP-9 stromal cells. Expression is relative to *Hprt*. Data in all panels are representative of at least 3 independent experiments. * P<0.05, **P<0.01, ***P<0.001 (unpaired, two-tailed Student's *t*-test).

Osteoblast- and hematopoietic-derived IL-7 is not required for B-lymphopoiesis

Previous studies suggested that osteoblasts form a specialized BM niche supporting B lineage cell development (Ding and Morrison, 2013; Visnjic et al., 2004; Wu et al., 2008; Zhu et al., 2007). Osteoblasts differentiated in vitro can express IL-7, and CXCL12 has also been detected in osteoblasts in vivo, albeit at very low amounts (Ding and Morrison, 2013; Sugiyama et al., 2006). Furthermore, a significant percentage of IL-7Ra$^+$ lineage-negative cells, of which ~8% are CLP (Figure S4A), is positioned near the endosteum (Ding and Morrison, 2013). Thus, we asked whether Ly6D$^+$ CLPs were preferentially distributed near the endosteum. To address this question, we quantified Ly6D$^+$ CLP distance to the nearest bone surface and found that more than 80% of these cells were positioned > 30 μm away from the endosteum (Figure S4B and C). We then asked if *Il7* was expressed in osteoblasts in vivo and in vitro. To address this question, we stained 25 μm-thick femur whole mount sections from *Il7* reporter mice or from control mice with anti-osteopontin or with anti-osteocalcin antibodies and found no evidence for *Il7* expression in osteoblasts in situ (**Fig. 4.4A**, and Figure S5A, respectively). In order to analyze *Il7* expression in osteoblasts in a quantitative manner we measured *Il7* mRNA expression in cultured osteoblasts from adult femur BM precursors, and from neonatal calvaria BM precursors, by quantitative PCR. We found that *Il7* expression is essentially undetectable in these cells, whereas osteocalcin (*Bglap*, a gene highly expressed in pre-osteoblasts and mature osteoblasts) was readily detected, and *Cxcl12* expression was detected at low levels (**Fig. 4.4B**, and Figure S5B), as expected (Ding and Morrison, 2013; Sugiyama et al., 2006). To convincingly rule out the possibility that an undetectable amount of Il7 expressed by osteoblasts contributes to B lymphopoiesis we conditionally deleted Il7 in osteoblasts using the *Col1a1* 2.3Kb promoter driven-cre recombinase approach (*Col2.3*-cre) (Liu et al., 2004). We found no evidence for a significant contribution of osteoblast-expressed IL-7 to B lymphopoiesis (**Fig. 4.4C**). Furthermore, we could not detect *Il7* expression in hematopoietic cell subsets (not shown), nor did *Il7* deletion from hematopoietic cells using the *Vav*-cre transgene resulted in measurable defects in B cell production (**Fig. 4.4D**).

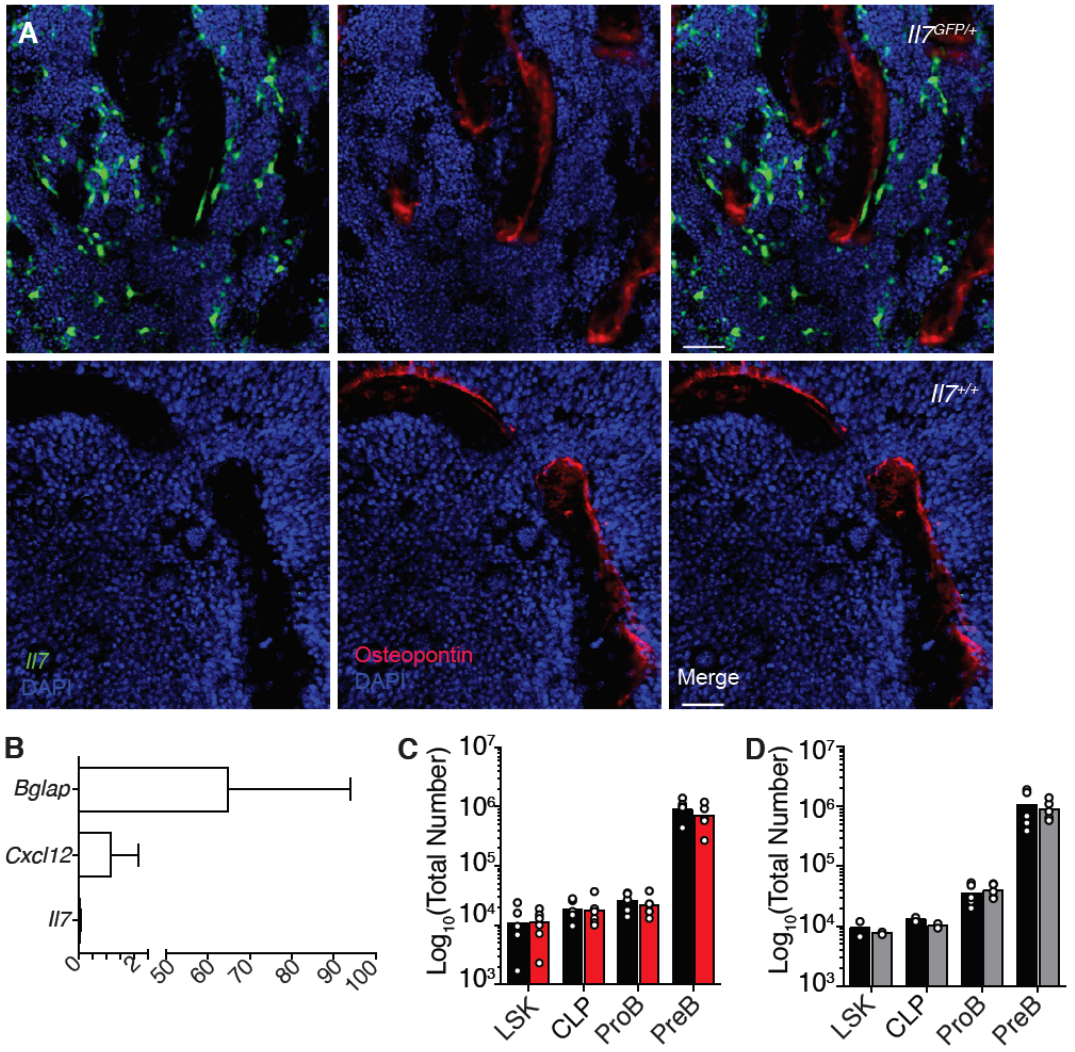


Figure 4.4| Osteoblast-derived *Il7* is not required for B lymphopoiesis. (A) Distribution of IL-7^{+} (green) and osteopontin^{+} (red) cells in BM. (B) *Bglap* (osteocalcin), *Cxcl12*, and *Il7* expression in osteoblasts by qPCR. Gene expression is relative to *Hprt*. (C) Total number of hematopoietic cell subsets in BM of *Il7*$^{fl/-}$;*Col2.3*-cre^{+} (red) and control littermates (black). (D) Total number of hematopoietic cell subsets in BM of *Il7*$^{fl/-}$;*Vav*-cre^{+} (gray) and control littermates (black). Bars indicate average, circles depict individual mice. Data in all panels are representative of 3-independent experiments, at least.

IL-7^{+} cells are a subset of BM CAR cells and are critical for B-lymphopoiesis

The fact that CXCR4 was critical for CLP positioning near or in contact with IL-7-producing reticular cells suggested that IL-7^{+} cells express

significant amounts of CXCL12. However, distinct stromal cell subsets were suggested to produce either CXCL12 or IL-7, but not both (Tokoyoda et al., 2004). To gain insight on the BM niches that support lymphoid lineage differentiation, we analyzed the cellular composition and distribution of IL-7^{+} cells in BM of *Il7*$^{GFP/+}$;*Cxcl12*$^{DsRed/+}$ double reporter mice. We carefully extracted and digested BM cells with collagenase (see Materials and Methods for details) and analyzed cells by flow cytometry. We found that 0.05±0.01% of total BM cells express *Il7* (**Fig. 4.5A**). Of these cells, 90.3±6.0% expressed *Cxcl12* at very high levels (**Fig. 4.5B**), and were large reticular cells distributed in BM parenchyma and perivascular areas (**Fig. 4.5D** and **E**). Occasionally, we could find extremely large and elongated IL-7^{+} cells adjacent to arterioles, which resembled a population of Nestin-GFP bright cells (Nestin^{peri}; data not shown) (Kunisaki et al., 2013). A few IL-7^{+} cells did not express *Cxcl12* (2.8±3.2%, **Fig. 4.5B** and **D**), and these cells were also large reticular cells distributed in parenchyma and perivascular areas (not shown). Although >97% of IL-7^{+} cells expressed *Cxcl12* (at intermediate and high amounts), only 61.5±3.9% of the CXCL12^{+} cells also expressed *Il7* (**Fig. 4.5C**).

CAR cells share functional similarities with a subset of mesenchymal progenitor cells that expresses Leptin receptor (LEPR; Omatsu et al., 2010; Zhou et al., 2014). Interestingly, the majority of IL-7^{+} cells (88.3±8.6%) expressed LEPR, (**Fig 4.5E**); and *Lepr*-cre driven tdTomato expression marked IL-7^{+} BM cells (**Fig. 4.5F**). Next, we asked if *Il7* expressed by LEPR^{+} cells was required for B lymphopoiesis. We found that conditional deletion of *Il7* from LEPR^{+} cells resulted in a significant reduction in Ly6D^{+} CLP in BM (**Fig. 4.5G**), and reduced pSTAT5a in these cells (**Fig. 4.5H and I**). Consequently, the number of developing B cell subsets was dramatically reduced (**Fig. 4.5J**), which led to peripheral B lymphopenia (**Fig. 4.5K**). Finally, deletion of *Il7* from mesenchymal progenitor cells using PRX1 (a transcription factor expressed in the limb bud mesoderm, *Prx1*-cre (Logan et al., 2002)) also led to a dramatic (and significant) reduction in developing B cell subsets in BM (Figure S6).

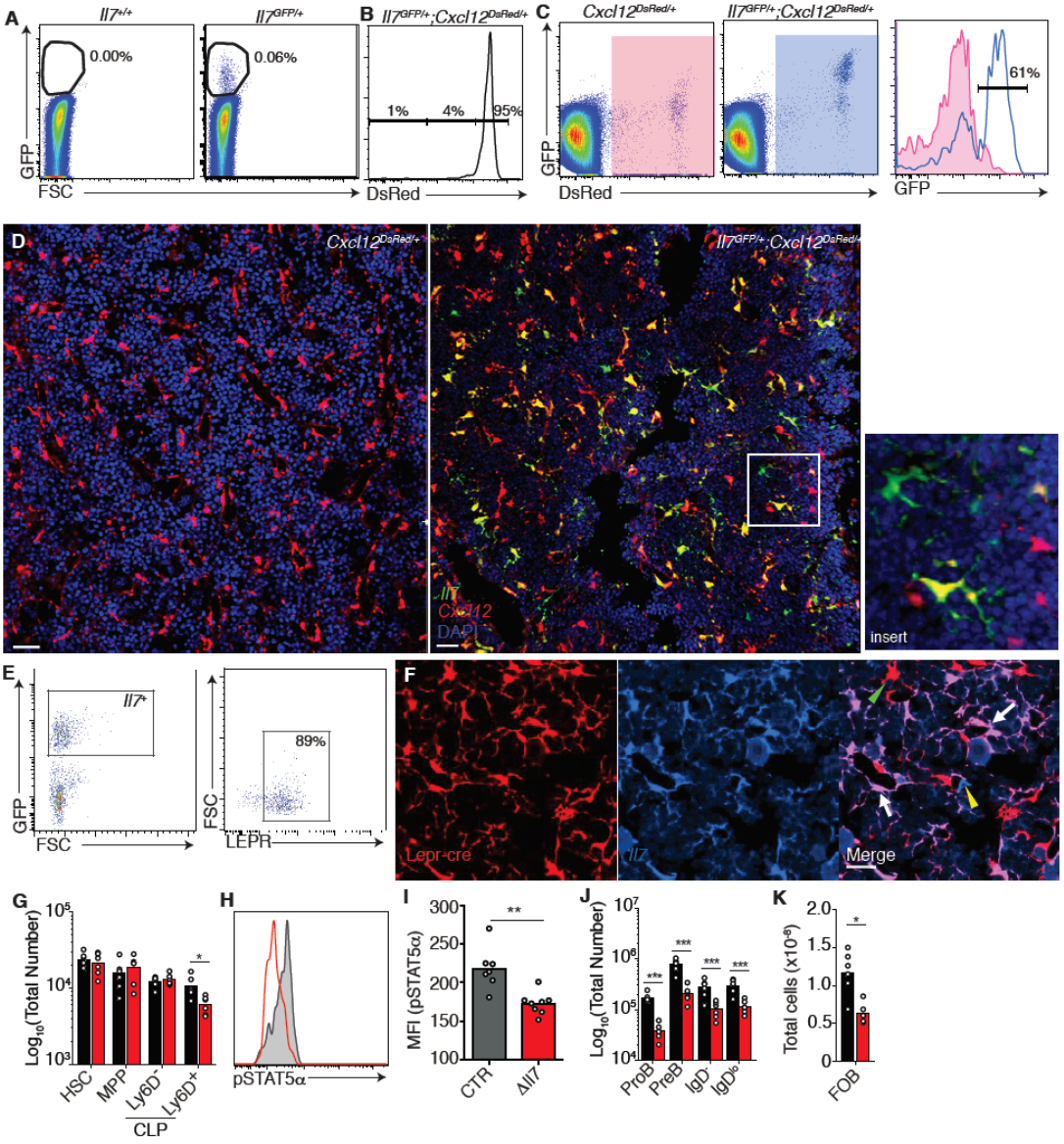


Figure 4.5| Most IL-7+ cells are a subset of CXCL12-abundant reticular cells. (A) Frequency of IL-7^{+} cells in BM. (B) *Cxcl12* expression in IL-7^{+} CD45^{-} cells. (C) *Cxcl12* and *Il7* expression in CD45^{-} cells (left and middle). *Il7* expression in *Cxcl12*^{Hi} cells (right). (D) 25 μm-thick section of *Il7*$^{GFP/+}$;*Cxcl12*$^{dsRed/+}$ mouse femur stained to detect IL-7^{+} (green) cells. CXCL12^{+} cells were directly visualized. Scale bar is 50 μm. Insert, example of an IL-7^{+} CXCL12^{-} cell, and of an IL-7^{+} CXCL12^{+} cell. (E, Left) gated IL-7^{+} cells; (right) LEPR expression in gated IL-7^{+} cells. (F) Lineage mapping of *Il7* expressing cells using *Lepr*-cre transgenic mice. 8 μm-thick femur section of *Lepr*-cre^{+};*Rosa26*$^{tdtomato/+}$ *Il7*$^{GFP/+}$ mice. Scale bar is 50 μm. Arrow and arrowhead indicate *Lepr*-cre-derived IL-7^{+} cells and *Lepr*-cre-derived IL-7^{-} cells respectively. Yellow arrow indicates IL-7^{+} *Lepr*-cre^{-} cells. (G) HSC, MPP, and CLP number in BM of *Lepr*-cre^{+};*Il7*$^{f/fl}$ (red) and *Lepr*-cre^{-};*Il7*$^{f/fl}$ (black) mice. (H) Histogram of pSTAT5a in Ly6D^{+} CLPs from *Lepr*-cre^{+};*Il7*$^{f/fl}$ (blue) and *Lepr*-cre^{-};*Il7*$^{f/fl}$ (filled). (I) pSTAT5a geometric mean intensity in Ly6D^{+} CLPs in *Lepr*-cre^{-};*Il7*$^{f/fl}$ mice (gray) and in *Lepr*-cre^{+};*Il7*$^{f/fl}$ (red) mice. (J and K) Developing B cell numbers in BM (J); splenic B cells (K) in *Lepr*-cre^{+};*Il7*$^{f/fl}$ (red) and *Lepr*-cre^{-};*Il7*$^{f/fl}$ (black). In panels G, I, J and K, bars indicate mean,

circles depict individual mice. Data are pooled from 3 independent experiments. * P<0.05, **P<0.01, ***P<0.001 (unpaired, two-tailed Student's *t*-test).

Endothelial cell-derived IL-7 contributes to B lymphopoiesis

A previous study indicated that some IL-7^{+} cells in BM express endothelial cell markers (Hara et al., 2012). Likewise, we found that a smaller fraction (20.6±7.2%) of IL-7^{+} cells expressed CD31 (PECAM-1), Sca1, and VE-Cadherin (**Fig. 4.6A** and **B**). To rule out the possibility of an in vitro staining artifact, we analyzed IL-7^{+} cells in relation to BM endothelial cells by confocal microscopy. We found that in some BM sinusoids, CD31 and CD144 (VE-Cadherin) co-localize with GFP expression driven by *Il7* (**Fig. 4.6B**). We then sorted CD144^{+}CD31^{+} CD45^{-} Ter119^{-}BM endothelial cells, and LEPR^{+} CD144^{-} CD31^{-} CD45^{-} Ter119^{-} MSPC, and compared *Il7* and *Cxcl12* expression by qPCR. We found that even though *Il7* (and *Cxcl12*) was mostly expressed by MSPC, *Il7* expression could also be readily detected in BM endothelial cells (**Fig. 4.6C**). Importantly, *Il7* deletion from endothelial cells using a *Tie2*-Cre transgene resulted in a small, selective, but significant reduction in Pre-B cell numbers in BM (**Fig. 4.6D**), suggesting that Pre-B cell proliferation is sensitive to endothelial sources of IL-7.

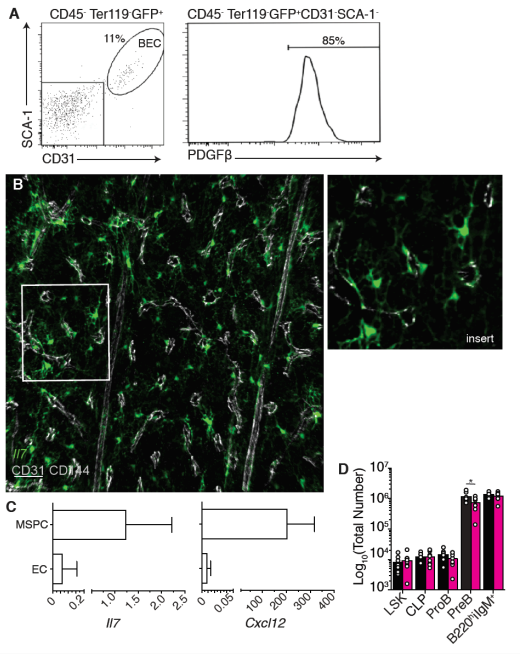


Figure 4.6| Endothelial cell-derived IL-7 contributes to B cell development. (A) Flow cytometry analysis of Sca1 and CD31 expression in IL-7^{+} BM cells. Blood endothelial cells (BECs). (B) *Il7* co-localization with CD31 and VE-Cadherin. Scale bar: 50 μm. (C) *Il7* and *Cxcl12* expression in FACS sorted CD45^{-}Ter119^{-} CD144^{-}CD31^{-}LEPR^{+} MSPC and endothelial cells (ECs, CD45^{-}Ter119^{-}CD144^{+}CD31^{+}LEPR^{-}). Gene expression is relative to *Hprt*. Bars indicate average ± SD of 3-independent cell sorting experiments. (D) Total number of hematopoietic cell subsets in BM of *Il7*$^{Fl/-}$;*Tie2*-cre^{+} (red) and control littermates (black). Bars indicate average, circles depict individual mice. * P<0.05, (unpaired, two-tailed Student's *t*-test).

IL-7$^+$ cells are mesenchymal progenitor cells

CAR cells are mesenchymal progenitor cells that can differentiate into multiple cell lineages including osteoblasts, osteocytes, chondrocytes and adipocytes, and possibly stromal cells (Omatsu et al., 2010). As most IL-7$^+$ cells form a subset of CAR cells it raised the possibility that IL-7$^+$ cells also overlap with mesenchymal progenitor cells. Alternatively, IL-7$^+$ CAR cells could represent a differentiated mesenchymal subset that no longer retains multipotent differentiation potential. To distinguish between these possibilities we traced cell lineages that differentiated from IL-7$^+$ cells by crossing *Il7*-cre transgenic mice (Repass et al., 2009) with *Rosa26*^{YFP} mice (YFP expression driven by Rosa26 repressed by a loxP-flanked transcriptional repressor), and examined the cellular composition and distribution of YFP$^+$ cells in BM. We found that a large fraction of bone-lining osteocalcin$^+$ osteoblasts were YFP$^+$, and a few bone-embedded osteocytes were also YFP$^+$ (**Fig. 4.7A**). To determine if adipocytes could differentiate from IL-7$^+$ cells, we induced adipogenesis by sub-lethal irradiation (Bryon et al., 1979) and examined femurs and tibias of *Il7*-cre$^+$;*Rosa26*^{YFP} mice for YFP expression in perilipin$^+$ adipocytes. We found that irradiation-induced adipogenesis occurred partly from IL-7$^+$ cells (**Fig. 4.7B**), while adipocytes expressed marginal amounts of *Il7* and high amounts of adipocyte-specific adiponectin and perilipin (**Fig. 4.7C**). Combined, these data show that IL-7$^+$ CAR cells are mesenchymal progenitor cells with multilineage differentiation capacity. These data also showed that *Il7* expression is down-regulated when IL-7$^+$ CAR cells undergo differentiation.

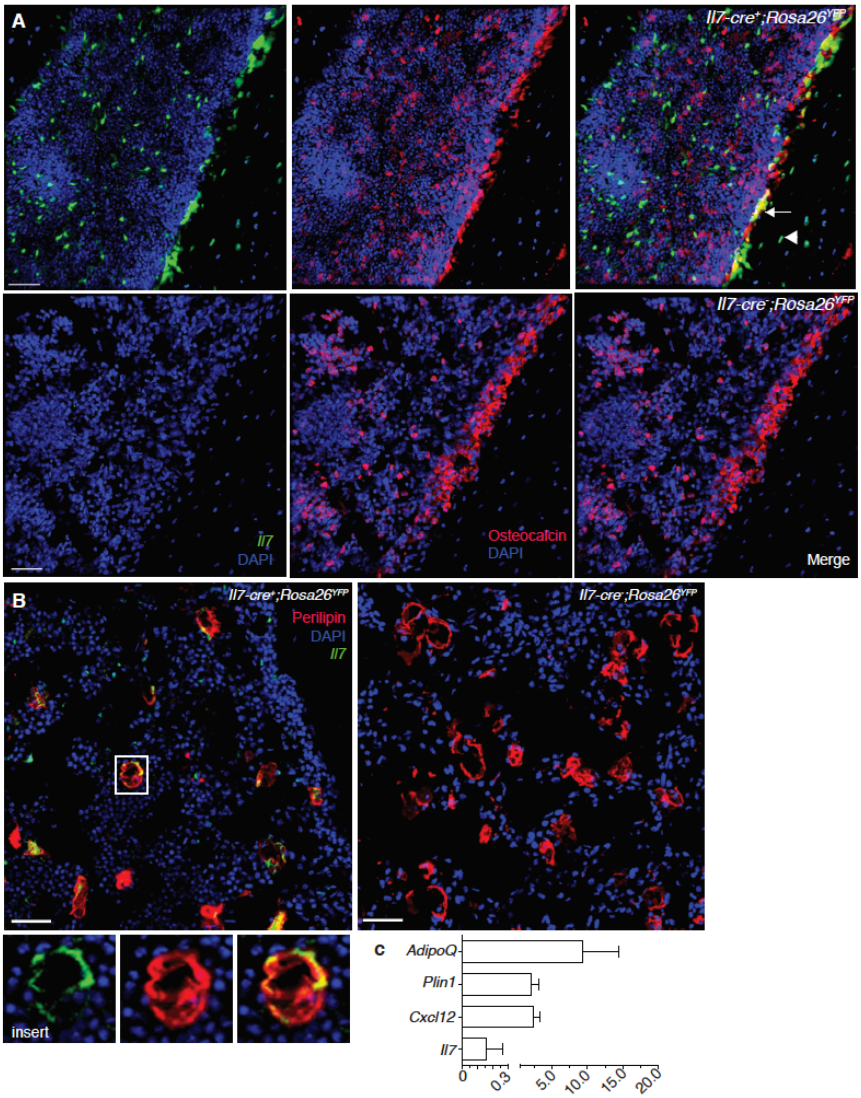


Figure 4.7| IL-7+ BM cells are mesenchymal progenitor cells. (A) Osteoblasts and osteocytes differentiate from IL-7$^+$ cells. Arrow indicates osteoblasts, arrowhead indicates osteocyte. (B) adipocytes differentiate from IL-7$^+$ cells. (A and B) 25 μm-thick section of *Il7*-cre$^+$;*Rosa26*$^{YFP/+}$ mouse femur (top panels), or of control mouse femur (bottom panels), stained to detect osteocalcin$^+$ cells (red, A); perilipin$^+$ cells (red, B). YFP$^+$ cells (green). (C) *Adipoq*, *Plin1*, *Cxcl12*, and *Il7* mRNA expression in adipocytes. mRNA expression is relative to *Hprt*.

4. Discussion

Here we showed that the BM niche formed by CXCL12$^+$ cells represents a common niche for hematopoietic progenitors at multiple stages of differentiation. CXCR4 was critically required in hematopoietic progenitor

cells for positioning in the vicinity of IL-7^{+} cells in BM, and that proximity to IL-7 enables optimal IL-7R signaling and B cell developmental progression. The majority of IL-7^{+} cells form a subset of mesenchymal progenitor CAR cells capable of multilineage differentiation, and a minor fraction of IL-7^{+} cells are BM sinusoidal endothelial cells.

HSC require short-range extrinsic factors, such as angiopoietin, SCF, and CXCL12 amongst several others, produced by rare mesenchymal progenitor cells in BM for their long-term maintenance. SCF-producing mesenchymal progenitor cells overlap entirely with CAR cells (Ding et al., 2012), and IL-7 expression marks the mesenchymal progenitor subset that expresses the highest amounts of CXCL12. Furthermore, Nestin-expressing mesenchymal cell subsets (namely Nestin^{Bright} and Nestin^{Dim}) are also critical components of HSC niches in BM, and these cells also express essential HSC maintenance factors and largely overlap with IL-7^{+} and IL-7^{-} CAR cells. Importantly, Nestin^{Bright} cells play a critical role in HSC quiescence and long-term maintenance most likely because these cells express higher amounts of HSC maintenance factors (e.g. angiopoietin, SCF) than Nestin^{Dim} cells (Kunisaki et al., 2013). Importantly, Nestin^{Bright} cells also express significantly higher amounts of CXCL12 and IL-7 (Kunisaki et al., 2013). As HSC are highly dependent on CXCR4 signaling for quiescence and long-term maintenance (Nie et al., 2008; Sugiyama et al., 2006), our studies suggest that HSC quiescence and lymphopoiesis are distinct cellular decisions controlled by highly overlapping mesenchymal niches.

Mesenchymal progenitor cells also respond to long-range signals, such as cytokines and hormones, which regulate mesenchymal progenitor cell activity. For example, adrenergic signals controlled by circadian rhythms are sensed by beta 3 adrenergic receptors expressed on mesenchymal progenitor cells and reduce CXCL12 production (Mendez-Ferrer et al., 2008). Consequently, HSC are displaced from HSC niches and exit the BM in a circadian manner (Mendez-Ferrer et al., 2008). Although the physiological role for HSC recirculation remains unknown (Wright et al., 2001), we suggest that circadian fluctuations in CXCL12 production enable HSC to divide periodically, and possibly allow a few HSCs to differentiate into MPP. As our data showed that hematopoietic cell lineages are differentially sensitive to CXCR4 (lymphoid lineages being more dependent on CXCR4 than myeloid and erythro-megakaryocyte lineages, see Fig. 1B and 1D), we suggest that

circadian fluctuations in CXCR4 signaling in MPP presumably alters their positioning in relation to mesenchymal progenitors, and thus enables the production of distinct hematopoietic lineages in a periodic manner.

Previous studies implicated osteoblasts as an essential component of BM niches supporting B lymphopoiesis (Visnjic et al., 2004, Wu et al., 2008, Zhu et al., 2007). Furthermore, a recent study found that conditional deletion of *Cxcl12* in osteoblasts using the *Col2.3*-cre transgene, led to a small but significant reduction in BM CLP that did not cause Bl ymphopenia (Ding and Morrison, 2013). Our findings are in apparent contrast with these studies, but are in agreement with the finding that the low *Cxcl12* expression in osteocalcin^{+} osteoblasts is not required for B lymphopoiesis (Greenbaum et al., 2013). However, the strategies used for assessing the role played by osteoblasts in lymphopoiesis bear considerable limitations. For example, the use of conditional approaches for inducing osteoblast apoptosis result in synchronized cell apoptosis within BM that may not be efficiently cleared by phagocytic macrophages. Consequently, the release of intracellular components, such as genomic and mitochondrial DNA, can elicit a significant inflammatory response, including the production of IL-1, TNFα, and type I IFNs (Chen and Nunez, 2010). In turn, inflammatory signals not only reduce B lymphopoiesis (Ueda et al., 2004, Ueda et al., 2005), but also affect HSC numbers (Glatman Zaretsky et al., 2014), and these changes were evident in both studies (Visnjic et al., 2004; Zhu et al., 2007). Given that most, if not all, studies using cell ablation approaches did not include appropriate control experiments (e.g. ablation of an irrelevant and numerically equivalent cell population), their conclusions remain subject to such limitations. Using an *Osterix*-driven cre recombinase approach, another study reported that Gα_s protein signaling in osteoblasts regulates B lymphopoiesis (Wu et al., 2008). However, CAR cells also express *Osterix* (Omatsu et al., 2010), and *Osterix*-driven cre recombinase expression marks mesenchymal progenitor cells (Greenbaum et al., 2013; Mizoguchi et al., 2014), which implicates Gα_s protein signaling in MSPC as a significant regulator of B-lymphopoiesis. Furthermore, although the *Col2.3*-cre approach has been widely used for the genetic manipulation of osteoblast-lineage cells, it should be noted that *Col1a1* expression has also been detected in MSPC (Ding et al., 2012; Kunisaki et al., 2013; Mendez-Ferrer et al., 2010). Therefore, it is possible that the *Col2.3*-cre transgene is already active at the MSPC stage, albeit

inefficiently.

B cells develop through multiple developmental stages in which entry and exit from cell cycle and Rag protein expression are tightly controlled. Interestingly, some evidence supports the notion that this process relies on distinct BM niches. Specifically, the large majority of Pre-proB cells have been found in proximity to CAR cells, Pro-B cells were mostly found near IL-7^{+} cells that did not express CXCL12, and Pre-B cells seem to localize near poorly defined Galectin^{+} cells (Mourcin et al., 2011; Tokoyoda et al., 2004). B-lineage cell movement towards and away from IL-7^{+} cells has been proposed to regulate Rag protein expression and activity (Clark et al., 2014, Johnson et al., 2008, Ochiai et al., 2012). Our results indicated that endothelial-derived *Il7* contributed primarily to Pre-B cell development. Our data also clearly demonstrated that *Il7* is mostly expressed by a subset of CAR cells, while only very few IL-7^{+} cells were CXCL12^{-}. In fact, our data suggests that *Il7* is expressed at very low amounts as we could barely visualize IL-7^{+} cells without signal amplification with anti-GFP antibodies. The fact that supraphysiological levels of IL-7 lead to the development of Pre-B cell leukemias in mice (Fisher et al., 1993) also supports the notion that IL-7 must be expressed in low amounts in BM. Given the notorious difficulty we, and others (Mazzucchelli et al., 2009), had in visualizing IL-7^{+} cells in situ, it is possible that the previous studies that identified IL-7^{+} cells in BM (Tokoyoda et al., 2004; Tokoyoda et al., 2009) were able to visualize IL-7^{+} CXCL12^{-} cells only. Thus, it will be important to determine the distribution of early B cell developmental subsets and of memory T cell subsets relative to such distinct IL-7^{+} cell subsets.

In summary, our studies revealed that IL-7 is produced in BM by a rare and heterogeneous cell subset composed of mesenchymal cells with multilineage differentiation potential, and by a few sinusoidal endothelial cells. The majority of IL-7^{+} cells expressed the highest amount of CXCL12 in BM. Consequently, MPP and CLP rely on CXCR4 signaling for positioning in proximity to IL-7^{+} cells, which is essential for accessing limiting IL-7 and for receiving sufficient IL-7R signaling. Although IL-7 is essential for B and T cell development in adult mice (Carvalho et al., 2001; von Freeden-Jeffry et al., 1995), and likely in adult humans (Bendall et al., 2014, Parrish et al., 2009), NK cell development is mostly dependent on IL-15 (Kennedy et al., 2000). NK cells also developed poorly from CXCR4-deficient MPP and CLP, and CAR cells

express IL-15 (Noda et al., 2011). Combined, these data demonstrate that the control of cell positioning in BM niches is an essential checkpoint in lymphopoiesis as it enables hematopoietic precursor access to limiting lymphoid-instructive cytokines. The clear similarities that we have shown between lymphoid-supportive niches and HSC niches in BM favor a model in which HSC maintenance and multilineage differentiation are distinct cell lineage decisions controlled by overlapping BM niches. The functional, evolutionary, advantage(s) for the local control of HSC maintenance and multilineage differentiation remain(s) to be determined.

Supplementary Figures

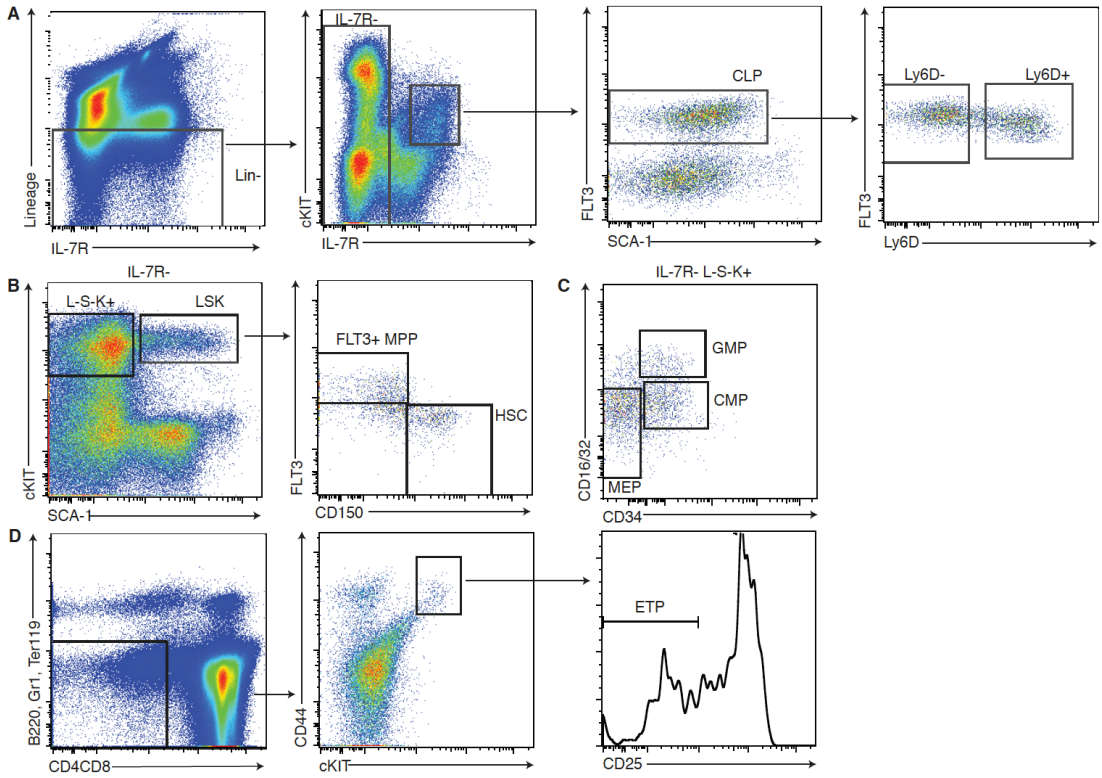


Figure S1| Gating strategy for analyzing HSCs and hematopoietic progenitor cell subsets. (A) Common lymphoid progenitor subsets. (B) Long-term HSC and MPP. Cells shown were gated on the indicated live ($DAPI^-$) LSK gate. (C) MEP, CMP and GMP. Cells shown were gated on live ($DAPI^-$) $IL7R^-$ $L^-S^-K^+$ gate. (D) ETP. Data are representative of more than 10 independent experiments.

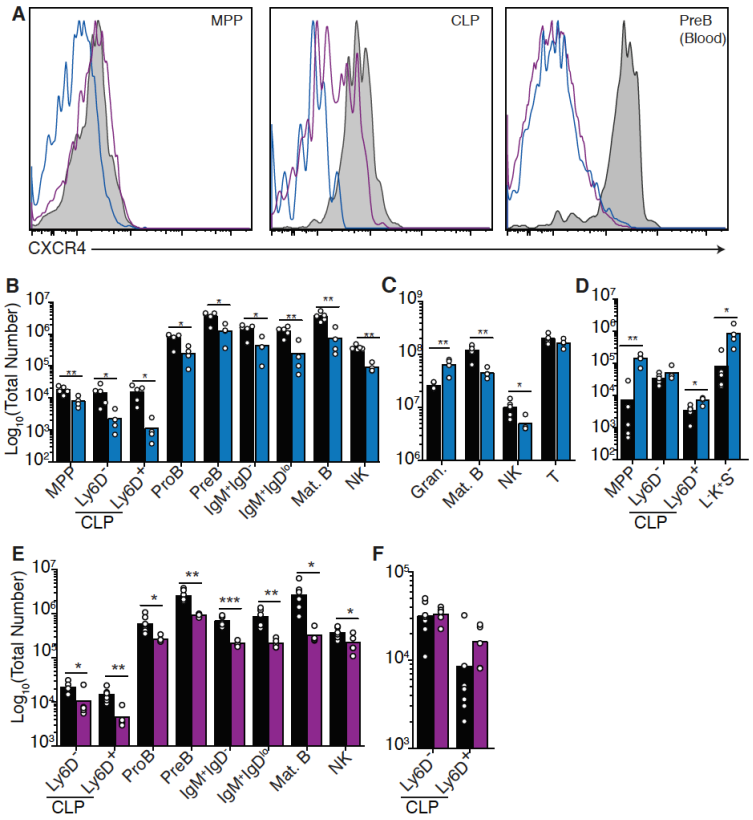
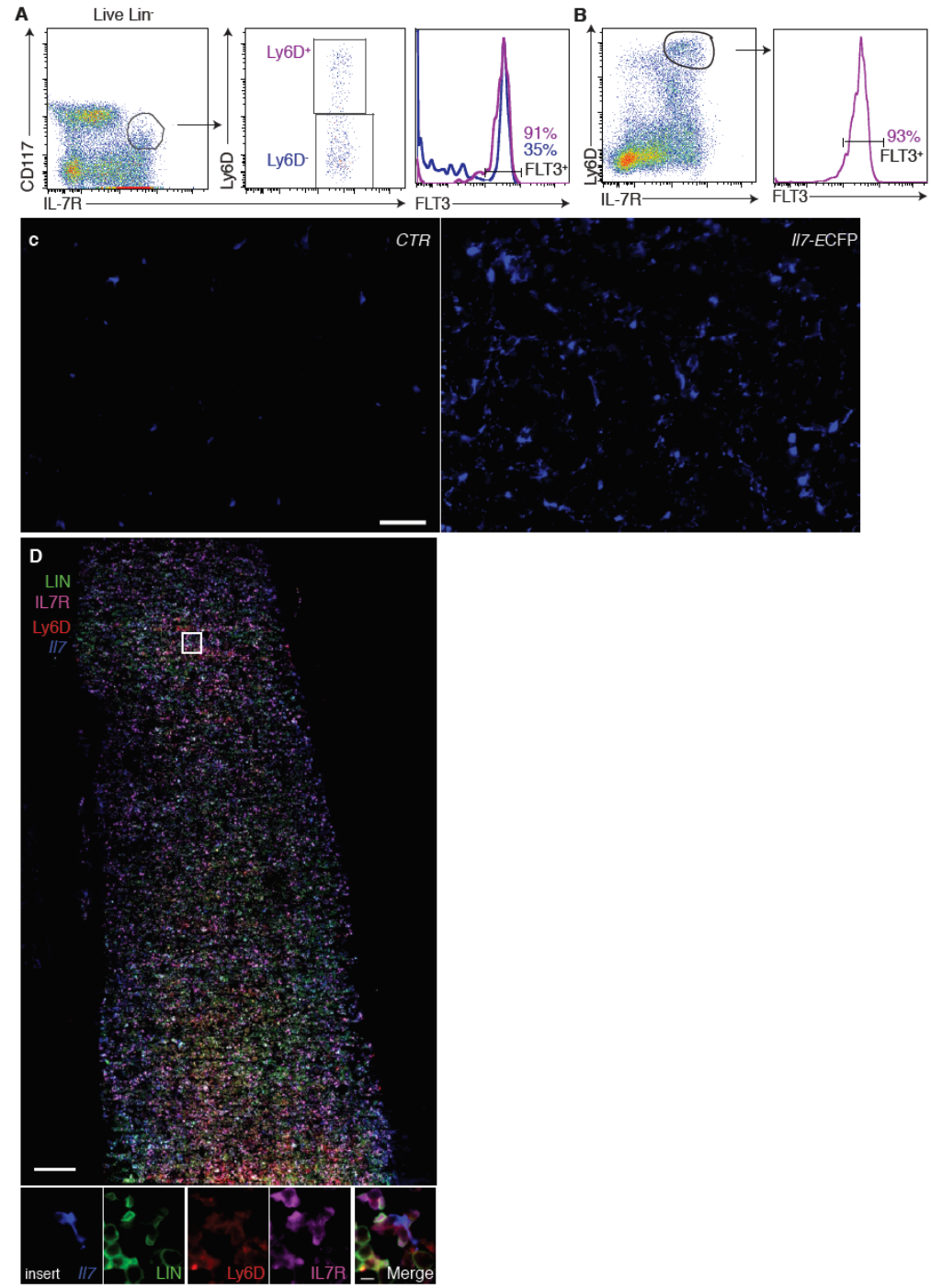


Figure S2| CXCR4 deficiency at MPP and CLP stage reduces lymphoid celldifferentiation. (A) Histogram of CXCR4 expression in gated MPP (left), CLP (middle), and Pre-B (right) cells isolated from control (filled), *Flk2*-cre^{+};*Cxcr4*$^{Fl/Fl}$ (blue), and *Il7ra*$^{cre/+}$;*Cxcr4*$^{Fl/Fl}$ (pink) mice. Data are representative of more than 3 independent experiments. (B-D) Analysis of *Flk2*-cre^{+};*Cxcr4*$^{Fl/Fl}$ (blue) and *Flk2*-cre^{-};*Cxcr4*$^{Fl/Fl}$ (black)mice. (B) Number of hematopoietic progenitor cell subsets, developing B cell subsets, and NK cells in the femur and tibia. (C) Total number of granulocytes (gran.), mature B cells, NK and T cells in combined primary and secondary lymphoid organs, peritoneal cavity, and in liver. D, Number of hematopoietic cell subsets in the spleen. (E and F) Analysis of *Il7ra*$^{Cre/+}$;*Cxcr4*$^{Fl/Fl}$ mice (purple) and *Il7ra*$^{Cre/+}$;*Cxcr4*$^{+/+}$ mice (black). (E) Number of hematopoietic progenitor cell subsets, developing B cell subsets, and NK cells in the femur and tibia. (F) Number of CLP subsets in the spleen. Bars indicate mean; circles depict individual mice. Data are pooled from at least 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001 (unpaired, two-tailed Student's *t*-test).



	Freq. (%)
LIN- IL7R+ Flt3+ cKitint Sca-1+Ly6D+ (FACS)	0.03
LIN- IL7R+ Ly6D+ (FACS)	0.08
LIN- IL7R+ Ly6D+ (IF)	0.07 (11/16064)

Figure S3| Strategy for in situ detection of Ly6D+ CLP cells. (A) FLT3 expression in live (DAPI-) lineage (lin-) negative BM cells gated on Ly6D+ and Ly6D- cells. (B) FLT3 expression in total Ly6D+ and IL7Ra+ BM cells. Left panel shows Lin- gated BM cells. Right panel shows FLT3 expression in gated population. (C) Distribution of IL-7+ cells in femur 7-μm sections stained with anti-GFP antibodies. Left, control (CTR); right, *Il7*-ECFP transgenic. (D) 7-mm femur section of *Il7*-ECFP transgenic mice stained with anti-GFP (blue), lineage antibody cocktail

(green), anti-IL-7Rα (purple), and with anti-Ly6D antibodies. Scale bar is 200 μm. White box in large panel indicates insert; individual fluorescence channels of magnified inserts are shown below. Data are representative of more than 10 independent experiments. Table, cell frequencies determined by immunofluorescence (IF) and by flow cytometry (FACS).

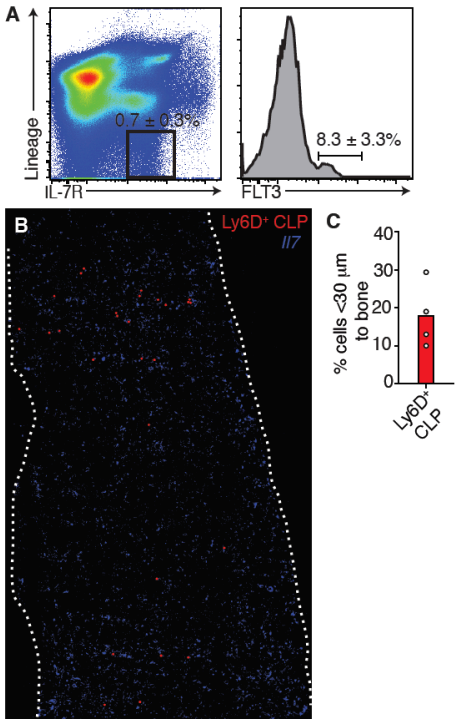


Figure S4| CLP gating strategy used by Ding et al 2, and CLP distribution in situ. (A) Left panel, frequency of total IL-7Rα$^+$ Lin$^-$ cells in BM. Right panel, FLT3 expression in gated IL-7Rα$^+$ Lin$^-$ cells. Numbers indicate average ± SD (n=8 mice pooled from 3 independent experiments). (B) Distribution of Ly6D$^+$ IL-7Rα$^+$ Lin$^-$ CLP and IL-7$^+$ cells in a 7 μm-thick section of *Il7*-ECFP transgenic femur. Dotted line indicates bone surface. Scale bar is 200 μm. (C) Frequency of Ly6D$^+$ IL-7Rα$^+$ Lin$^-$ CLP positioned at <30 μm from bone surface. Bars indicate average, circles depict individual mice. Data was pooled from 3 independent experiments.

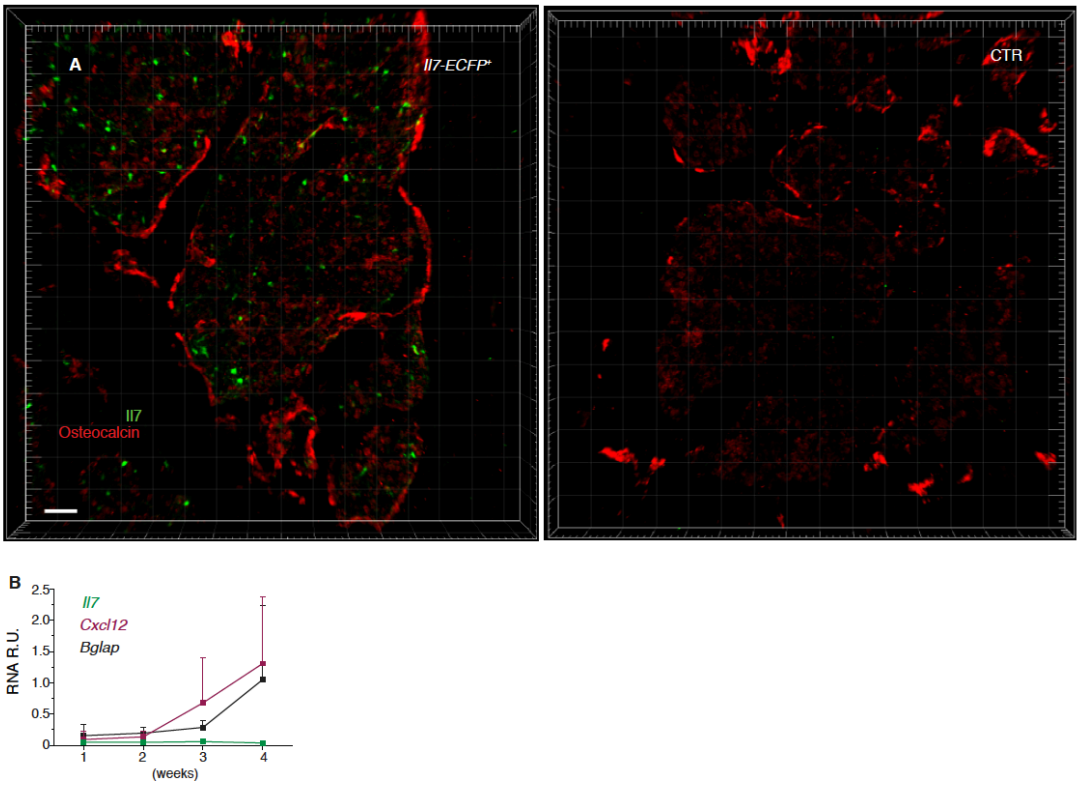


Figure S5| Distribution of IL-7^{+} cells in relationship with osteoblasts. (A) Distribution of IL-7^{+} cells and osteocalcin^{+} cells in 25 μm-thick femur whole mount. Scale bar is 50 μm. (B) *Il7*, *Cxcl12*, and *Bglap* expression in neonatal-derived calvaria osteoblasts cultured over a 4 week period (x-axis). Expression is relative to *Hprt* (y-axis). Lines indicate average ± SD of 3-independent experiments.

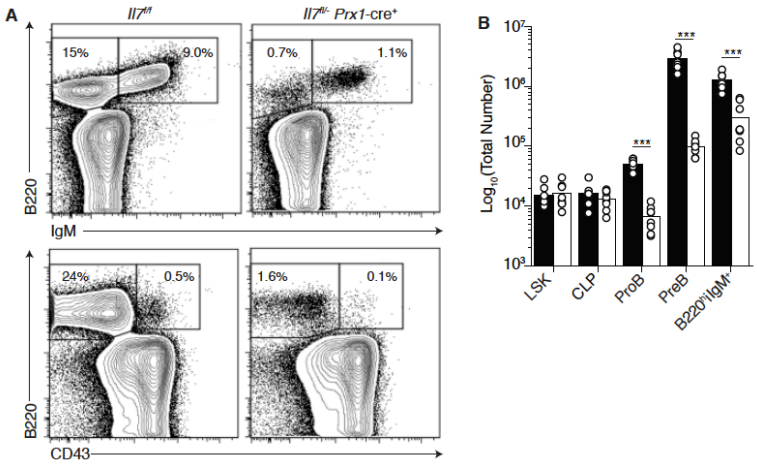


Figure S6| IL-7 expressed by MSPC is critical for B lymphopoiesis. (A) Flow cytometric analysis of BM from 4-wk-old *Il7*$^{Fl/-}$;*Prx1*-cre^{+} and control littermate mice. Numbers indicate the percentages of each fraction. (B) Absolute numbers of indicated cells in the femur and tibia. Bars indicate average, circles depict individual mice. *** p <0.001 (unpaired, two-tailed Student's *t*-test).

Chapter 5

Discussion

Chapter 5 | Discussion

The experimental data was discussed throughout chapters 3 and 4. Therefore, the following discussion aims to puzzle together the data, highlighting the novel findings, the open and/ or unanswered questions on defining/ characterizing hematopoietic differentiation niches, with emphasis on the B lymphopoiesis niche and on the mechanisms regulating the positioning of hematopoietic progenitors within the niche.

1. Re-Defining the Hematopoietic Niche

Transcription factors dictate cell lineage choices, but the signals controlling their expression and/or activity remain poorly understood. Therefore, it was initially hypothesized that HSC reside within a niche. Over the last decade, the effort of many research teams shed light on defining which cell types regulate the HSC niche. However, how the niche constituents are organized within bone marrow, and to what extent they interface with each other is still poorly understood. Moreover, the hematopoietic output is diverse, and several different cues are required to generate the different blood lineages. Furthermore, ablation of CXCR4 function on HSC themselves affected more B lymphopoiesis than myelopoiesis (Nie et al., 2008), suggesting that the positional cues are fundamental for lineage differentiation.

CXCR4 /CXCL12 pathway is the major organizing chemokine/ chemokine receptor in bone marrow. We (Beck et al., 2014) and others (Pereira et al., 2009, Nie et al., 2008, Nie et al., 2004) have shown that B lymphopoiesis proceeds normally in the absence of CXCR4. Deficiency in CXCR4 after commitment to the B lymphoid lineage accelerates the egress of developing Immature B cells to the periphery, but not the egress of Pro and Pre-B cells at the same extent. Another retention mechanism may act on Pro and Pre-B cell stages to maintain them within the bone marrow parenchyma and thus, further work is required to define this CXCR4-independent retention of Pro and Pre-B cell stages. Pro and Pre-B cells might be incapable to transendothelial migration.

Immune cell migration and chemoattraction are mostly dependent on $G\alpha_i$ protein coupled receptors (Cyster and Schwab, 2012). Interestingly, when we ablated $G\alpha_i$ function in developing B lymphocytes, B lymphopoiesis proceed normally in bone marrow, being immature B cell egress accelerated albeit to a less extent due to the lack of the egress active mechanism. This is explained because $S1P_1R$/ S1P pathway is also affected by the lack of $G\alpha_i$ function in developing B cells. Even though bone marrow B lymphopoiesis proceeds normally in absence of $G\alpha_i$ function, immature B in the periphery show a survival defect, suggesting that GPCR function is required for the survival of immature B cells in the periphery.

Two major findings arise from these data. First, we revealed a passive egress mechanism, which we further characterized in (Beck et al., 2014). Second and most remarkably is that later stages of B lymphopoiesis are not critically dependent on positional cues, thus, challenging the concept of a differentiation niche, and favoring the initial niche concept where lineage differentiation occurs distantly to the maintenance HSC niche. A few studies have supported the existence of different hematopoietic niches, being HSC maintenance niche a distinct and separated entity to the B lineage differentiation niche (Ding and Morrison, 2013b, Zhu et al., 2007, Bai et al., 2007). The HSC niche have been described as perivascular and the B lymphoid niche to be close to endosteal regions and regulated by osteoblasts. These studies present some caveats. Their conclusions need to be rigorously determined on 1) the identity and abundance of the cell populations that are recombining and possible off-target effects, such as the deletion occurring in other cell types; 2) the efficiency of recombination in the targeted cell type; and 3) effects related to the kinetics of the induced deletion. It is important to retain that these considerations are critical to genetic models in which a specific cell population is ablated through *Cre*-mediated expression of a suicide gene (such as diphtheria toxin). In these models, the elimination of a cell population also affects its progeny, causing a compensatory feedback response in its precursor cells, or generating local inflammatory responses that may alter other cell populations secondarily (Mendez-Ferrer et al., 2015). Moreover, several *Cre* lines, such as *Col2.3-cre*, were incompletely characterized and instead of marking specific cell types,

may recombine in broader cell populations, leading to misunderstanding about which cell type is responsible for the phenotype observed.

The B lineage differentiation niche is not fully characterized. A strategy to define a lineage differentiation niche is to focus on factors critical for certain lineages, namely cytokines. Cytokines stimulate the survival and proliferation of hematopoietic stem and progenitor cells and also influence their lineage output. Whether lineage commitment occurs due to cell intrinsic mechanisms, such as fluctuating transcription factor networks, followed by a selective cytokine function, or is directly instructed by cytokines through the induction of lineage specific transcriptional programs has been technically challenging to demonstrate and distinguish. Adult murine B lymphopoiesis has been extensively reported as critically dependent on IL-7 (Carvalho et al., 2001, Dias et al., 2005, von Freeden-Jeffry et al., 1995, Vosshenrich et al., 2004, Vosshenrich et al., 2003). IL-7 is a soluble cytokine whose production is tightly regulated, implying that IL-7 consumption has to occur locally, i.e., close to its source. The arguments supporting the local consumption of IL-7 have been outlined in the discussion section of Chapter 4. IL-7 is required at the CLP stage to maintain the B lymphoid potential, arguing for an instructive role of IL-7 in B lymphopoiesis. The requirement for local consumption of an instructive cytokine in B lymphopoiesis allows to better identify and characterize a differentiation niche, namely, the B lymphopoiesis niche and molecular mechanisms regulating it.

In chapter 4, we showed that B lineage primed CLP localize preferentially close to IL-7-expressing BM cells. Moreover, CLP rely on the chemokine receptor CXCR4 signaling to position in proximity to IL-7-expressing cells, which is essential for accessing limiting IL-7 and for receiving optimal IL-7R signaling. Therefore, our studies revealed a contribution in vivo of a concerted action of chemokines and cytokines in the lineage differentiation of hematopoietic progenitors. Moreover, we demonstrated that different hematopoietic lineages have a differential requirement on CXCR4 at the progenitor level to access instructive cytokines, being lymphoid lineages highly dependent on CXCR4 on hematopoietic progenitors. Taken together, our data supports the model that in vivo cytokines and chemokines have an instructive role in hematopoiesis, and are major players in the hematopoietic niche.

Previous observations have reported that MPP represent a highly 'instructable' progenitor population whose lineage output can be reprogrammed in response to external cues, namely inflammatory cytokines (Maeda et al., 2005). Interestingly, lineage differentiated cells have been shown to modulate the hematopoietic niche by direct activation of mesenchymal progenitor cells, during stress responses, such as viral infections (Maeda et al., 2005), and by direct action on hematopoietic progenitors also during steady state (Mondal et al., 2011). Niche stromal cells express different cytokine receptors (Ding et al., 2012), and most likely they are capable of sensing extracellular cues and respond. As a consequence, the type of factors produced by these cells, namely instructive cytokines, may fluctuate, affecting lineage differentiation output. More studies are needed to understand how the hematopoietic niche senses external cues, under steady and stress states, and responds, and whether the responses affect lineage choice of progenitor cells.

2. The B lymphopoiesis niche as part of the HSC maintenance niche

Besides identifying it, we further characterized the B lymphopoiesis niche (Chapter 4), by thoroughly describing IL-7 sources in BM. IL-7-expressing cells share several features with the stromal cells implicated on the maintenance of HSC. Both cell types are shown to be mesenchymal progenitor cells (with adipogenic and osteogenic potential), which express LEPR, CXCL12. In addition, *Cxcl12*, *Il7*, and *Scf* mRNA was detected in Nestin-expressing mesenchymal stem cells (Mendez-Ferrer et al., 2010b). Our results provide a strong evidence of overlapping between lymphoid-supportive niches and hematopoietic stem cell niches in BM. Yet, the types of niche supporting cells remain unsolved. Different types of stromal cells and some hematopoietic cells, like macrophages (Chow et al., 2011) and megakaryocytes (Bruns et al., 2014), may constitute a niche unit. Further studies will have to determine whether different types of supporting cells constitute a niche unit. Considering that the niche has four (A, B, C, D) types of supporting cells, cell A, B and C express CXCL12. Cell A expresses SCF but

not IL-7; B expresses IL-7, but not SCF, and cell C expresses both IL-7 and SCF. Cell D expresses only IL-7. HSC may be positioned close to either cell A or cell C. CLP are either in close contact with cell B or C. It is possible that HSC are maintained by cell A and differentiate close to cell C. Therefore, it is imperative to determine the positioning of HSC (and MPP) relative to IL-7 cells, and to CLP, and determine whether IL-7$^+$ and SCF$^+$ stromal cells overlap. The identification of a HSC in close contact with the same IL-7 cell is strong indication but it is not sufficient to demonstrate an overlap between HSC maintenance and lineage differentiation niches. It will be necessary to demonstrate that HSC reside close to IL-7 cells and CLP differentiate into the B cell lineage, which is a challenging quest. For this purpose, G1 phase FUCCI reporter mice (Sakaue-Sawano et al., 2008, Hitoshi et al., 1991), in which FUCCI$^+$ LSK cells are enriched for HSC (Shangqin Guo, Yale Univeristy, personal communication), are being crossed with *cKit*$^{GFP/+}$ (Zhu et al., 2009). FUCCI$^+$ *cKit*$^{GFP/+}$ mice will be crossed with transgenic mice ubiquitously expressing the green-to-red, irreversibly photoconvertible protein KikGR mice (Tsutsui et al., 2005, Nowotschin and Hadjantonakis, 2009), and triple reporter mice will be crossed with IL-7-ECFP mice. Intravital 2-photon imaging of calvaria BM will allow to identify HSC in proximity to IL-7$^+$ cells. The area will be then exposed to violet light to induce photo-conversion. FACS analysis of the photo-converted cells will allow to identify which hematopoietic cells reside in proximity to HSC. Detection of Ly6D$^+$ CLP within the photo-converted cells would indicate that HSC and CLP reside in close proximity. A strategy to determine that the same supporting cell regulates HSC maintenance and lineage differentiation is to specifically ablate *Cxcl12* expression using *Il7*$^{cre/+}$ mice. Alteration in HSC pool and CLP would decree that CXCL12$^+$ IL-7$^+$ cells support HSC maintenance and B lymphopoiesis.

The fact that some supporting cells in the niche are MSC may also affect the lineage output, varying with the stages of the mesenchymal differentiation program. When the supporting cells are mainly primitive MSC, the niche maintains the HSC and supports B lymphopoeisis. We demonstrated that mature osteoblasts and adipocytes express low amounts of IL-7 then, with maturation of MSC into adipocytes and/or osteoblasts, the niche loses the capability of supporting B lymphopoiesis. This could account to the decreasing B lymphopoieisis with age.

The highlight of the hematopoietic niche (HSC maintenance and lineage-instructive niche) allows dissecting the poorly known structural organization of the bone marrow. For instance, it has been observed that bone marrow transits from red (hematopoiesis active) to yellow (hematopoiesis inactive) with age due to the accumulation of adipocytes, which have been implied as negative regulators of hematopoiesis (Naveiras et al., 2009). In the adult human, the red marrow is found mainly in the flat bones, such as the pelvis, sternum, cranium, ribs, and in the cancellous material at the epiphyseal ends of long bones (eg, femur and humerus). Why these changes in bone marrow structure occur is not completely understood. One possibility is that bone marrow parenchyma and the bone crosstalk to maintain the homeostasis of the marrow, and therefore regulate hematopoiesis. Shedding light on the mechanisms will be important to prevent/ cure pathologies such as osteoporosis. Moreover, hematopoietic failure is usually a consequence of overproduction of pro-inflammatory cytokines in pathologies such as chronic inflammatory diseases, hematological malignancies, and bone marrow failure syndromes (Bryder et al., 2001, Ishihara and Hirano, 2002). Hence, how the bone marrow comportment is regulated and how its regulation affects hematopoiesis are unanswered fundamental questions. It is feasible that HSC are the only important player in the regulation of hematopoiesis and therefore lineage output is only dependent on these multipotent cells. However, our studies show that multipotent hematopoietic progenitors are positioned in close proximity with specific types of bone marrow stromal cells, which argues that lineage differentiation is not a stochastic process determined exclusively at the HSC level, but instead the accessibility of multipotent progenitors to instructive cytokines is fundamental in hematopoietic output. It is still unknown whether multipotent hematopoietic progenitors regulate the niche and HSC maintenance and/or quiescence. If they do, do multipotent progenitors act directly on HSC in a paracrine fashion? Or do they directly crosstalk with the niche supporting cells? Addressing these questions will open important lines of investigation.

3. Implications of overlapping HSC maintenance and lineage differentiation in the same niche

If the same niche regulates both HSC maintenance and quiescence and lineage differentiation how do these fundamental but very different processes co-exist and are regulated is a major unknown.

HSC recirculation may affect lineage output. Circadian rhythms control HSC recirculation and leukocyte trafficking (Lucas et al., 2008, Mendez-Ferrer et al., 2008, Scheiermann et al., 2012). The fact that HSC recirculate antagonizes the notion that such important cell type has to be kept quiescent and protected within a niche. HSC release from the bone marrow is due to circadian fluctuation of CXCL12 (Mendez-Ferrer et al., 2009, Mendez-Ferrer et al., 2008). HSC recirculation due to circadian fluctuations may control lineage differentiation. On one hand, CXCL12 reduction attenuates CXCR4 signaling, which can have two outcomes. First, lower levels of CXCL12 reduce HSC quiescence, inducing the proliferation of the HSC, and as a consequence of less CXCR4 mediated retention, some HSC are released to the circulation, and home back to bone marrow when CXCL12 levels increase. When homing back to bone marrow, HSC may encounter different niches, which may dictate lineage choices. Second, lower CXCR4 signaling on MPP and CLP reduces IL-7 and presumably IL-15 consumption. Thus, the lack of this instructive cytokine impairs lymphoid differentiation, resulting in an increase of other lineages production. This model argues in favor of lineage decisions being made at the progenitors level but does not completely exclude the possibility of stochastic lineage decisions at the stem cell level, which leads to a second potential scenario.

Several studies have shown polyclonal output from HSC differentiation assays in vitro. In addition, HSC are also heterogeneous with respect to the ratio of myeloid to lymphoid cells that they generate upon transplantation into irradiated mice (Muller-Sieburg et al., 2004, Dykstra et al., 2007, Kent et al., 2009, Beerman et al., 2010, Challen et al., 2010, Morita et al., 2010) and with respect to their self-renewal potential upon transplantation (Ema et al., 2005, Morita et al., 2010, Benveniste et al., 2010). These findings suggest that HSC are imprinted with different lineage potential and thus, lineage differentiation/ commitment occurs at the stem cell level. The circadian

recirculation of HSC removes the stem cells from their maintenance niche, which along with possible sensing of extramedullary signals may affect lineage differentiation.

Besides HSC and hematopoietic multipotent progenitors, the bone marrow also harbors lineage-committed cells, such as developing B cells, recirculating mature B cells, NK cells, $CD8^+$ memory T cells, and granulocytes. Hence, how is the bone marrow organized to maintain and support all the different cell types? Our findings shed light on the existence of specialized hematopoietic niches, which combine multiple functions in order to maximize the efficiency of the hematopoietic output. In agreement with this possibility, computational modulation studies found that coupling together niche lineages allows the organism to regulate blood cell numbers as closely as possible to the homeostatic optimum (Szekely et al., 2014).

Bone marrow has to be organized to support the differentiation of different hematopoietic cells. Therefore, it is possible that different hematopoietic differentiation/ maintenance niches overlap. Moreover, fully differentiated hematopoietic cells, such plasma cells and memory T cells home back to bone marrow, and also require similar factors as developing hematopoietic cells. Indeed, memory T cells require IL-7 to survive and have been shown to reside in close proximity to IL-7 expressing bone marrow stromal cells (Sercan Alp et al., 2015, Mazzucchelli et al., 2009, Tokoyoda et al., 2009). IL-7 signaling in CD8 T cells must be intermittent and not continuous to promote cell survival (Kimura et al., 2013). CXCR4 is required to the homing of CD8 T memory cells to the bone marrow. Also, keeping the CD8 T cells in the marrow in a resting stage is important for secondary recalling (Chaix et al., 2014), and antigen is transported to bone marrow to be presented to CD8 T memory cells (Duffy et al., 2012). Hence, CD8 T cells may recirculate and home back to bone marrow when they require IL-7. Thus, T lymphocytes and CLP may compete for IL-7 in bone marrow, and in homeostasis, CD8 T cells may regulate/ modulate B lymphopoiesis by scavenging IL-7 from $Ly6D^+$ CLP. In addition, during inflammatory states, such as infections, B lymphopoeisis might be reduced because together with the decrease in the production of CXCL12 (Ueda et al., 2004), IL-7 is consumed by T cells residing in bone marrow and CLP are out competed.

Once formed the differentiated cells have to be exported from the bone marrow to the periphery. In Chapter 3, we demonstrate that CXCR4 signaling retains developing B cells in bone marrow parenchyma, but developing B cell egress from bone marrow was surprisingly independent of egress promoting chemoattractants sensed by PTX-sensitive GPCRs. These observations lead to a deeper analysis of the role of CXCR4 in B lymphopoeisis after B lineage commitment. CXCR4 signaling mediated an haptokinectic (dependent on $\alpha_4\beta_1$-VCAM1), amoeboid migration in developing B lymphocytes (Beck et al., 2014). Moreover, CXCR4 down-regulation enforced B cells to distribute into perisinusoidal e intrasinusoidal compartments and was critical for immature B cell egress from bone marrow, together with a passive egress mechanism (Beck et al., 2014). The mechanical forces responsible for the passive egress mechanism are not entirely clear, but such mechanisms likely involve shear stress, as the BM is a highly vascularized compartment. Besides the effects on hematopoietic cell egress, a potential effect of shear stress in the hematopoieitic niches, for instance, by affecting the extracellular matrix (Gattazzo et al., 2014), should be accessed.

4. A Model for B Lymphopoiesis in bone marrow

Our research highlighted how B lymphopoeisis occurs in vivo within bone marrow (**Figure 5.1**). Before commitment to B cell lineage, CXCR4 positions CLP in proximity of perivascular LEPR-expressing mesenchymal progenitor cells, which highly overlap with the HSC niche. This positioning allows an optimal consumption of IL-7, which is fundamental for the maintenance of the B lineage potential, most likely through the modulation of EBF levels (Dias et al., 2005). After commitment, CXCR4 retains developing B cells within bone marrow parenchyma by mediating their motility. CXCR4-mediated migration on B cells may allow the different stages of B cell development to encounter key factors. For instance, Pre-B cells have been described to reside in close proximity to Galectin-1-expressing bone marrow stromal cells, which do not express IL-7 (Mourcin et al., 2011). Interestingly, conditional *Il7* deletion from bone marrow endothelial cells did not affect B

cell progenitors but significantly reduced the number of Pre-B cells. These data suggests that IL-7 provided from different cellular sources may be important for different stages of B cell lymphopoeisis, which needs to be further determined.

At the immature stage, developing B lymphocytes significantly change their positioning within bone marrow, with up to 50% of immature B cells localizing within BM sinusoids in an $\alpha_4\beta_1$-VCAM-1 – and CB2 and $S1PR_3$ signaling-dependent manner (Pereira et al., 2009, Donovan et al., 2010). Immature B cells egress bone marrow by down-regulating CXCR4 by about twofold, simultaneously mechanical forces purge sessile B cells. It was described that CXCR4 down-regulation in immature B cells was antagonized by antigen-induced BCR signaling, presumably after negative selection has occurred (Beck et al., 2014). It will be valuable to analyze the cellular composition and biological significance of the BM niches that attract self-reactive immature B cells.

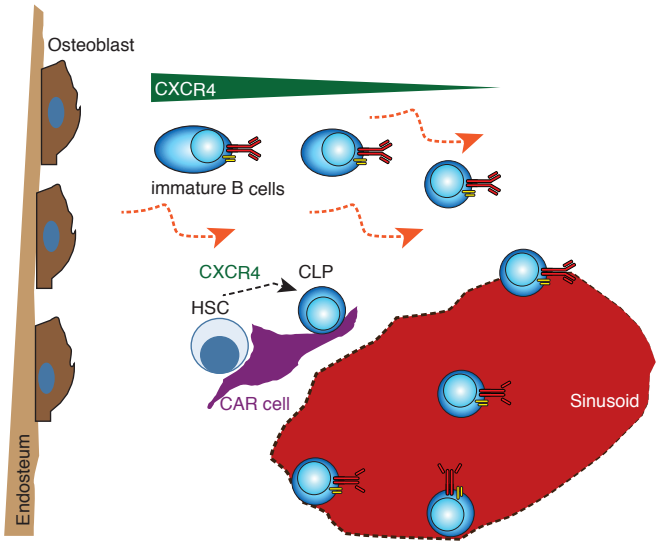


Figure 5.1| Model of positional regulation of B cell development. We found that CXCR4 mediates CLP positioning in the proximity to CAR cells, which express IL-7 and constitute most likely the B lymphopoiesis niche. The close proximity of CLP to CAR cells is critical for B lymphopoiesis because it allows optimal consumption of IL-7. Later on the development, immature B cells decrease CXCR4 expression and egress from BM.

Chapter 6

Conclusion and Future Perspectives

Chapter 6 | Conclusion and Future Perspectives

The major scientific contribution made in this thesis was the observation that hematopoietic multipotent progenitor differentiation is controlled by positional cues that enable the access to short-range lineage instructive cytokines produced by hematopoietic niches in bone marrow. Specifically, our research allowed us to conclude the following:

1. Hematopoietic multipotent progenitors require CXCR4 mediated chemoattraction to access short-range lineage-instructive cytokines produced by hematopoietic niches in bone marrow;
2. IL-7 acts as a short-range signal for CLP differentiation into B lymphocyte lineage;
3. IL-7-expressing cells are CXCL12-abundant reticular mesenchymal progenitor cells;
4. HSC maintenance and lymphopoiesis are controlled by overlapping bone marrow niches;
5. After commitment to B cell lineage, CXCR4-mediated haptokinectic migration retains developing B lymphocytes within bone marrow parenchyma.
6. Down-regulation of CXCR4 on immature B cells, and consequently reduction of their migration (active egress mechanism), together with action of mechanic forces (passive egress mechanism) allow the egress from bone marrow to the periphery where the final stages of B lymphopoiesis occur.

Our findings challenge not only the current model for HSC niche but also the current paradigm of early B lymphopoiesis, opening important lines of investigation. The positioning of developing B cells relative to IL-7-expressing cells needs to be further analyzed. In addition, it is fundamental to determine the molecular regulators of IL-7 expression. Ablation of $G\alpha_s$ in mesenchymal stem cells, such as CAR cells, osteoprogenitors, and osteoblasts severely affected pro- and pre-B cells in a fashion that could be

mitigated with IL-7 (Wu et al., 2008), suggesting that $G\alpha_s$ signaling may be a potential regulator of IL-7 expression in bone marrow.

Our data also changed the concept of immature B cell egress from bone marrow. The critical CXCR4 down-regulation before immature B cell egress was antagonized by antigen-induced BCR signaling, suggesting that immature B cell egress ensues after negative selection has occurred. These data creates a line of investigation of the cellular composition and biological significance of the BM niches that attract self-reactive immature B cells.

Understanding B lymphopoiesis is important but the actual breakthrough is to understand what determines the choice of a specific lineage pathway under homeostasis and how the niche responds to changes in homeostasis, such as inflammation and/or leukemia.

As far as I am concerned, a lot of effort should be invested in determining the mechanisms that control the niche size to avoid exhaustion of the stem cell pool and over proliferation of the multipotent progenitors. The niche is a dynamic unit, where all cell types must interact and communicate with each other. Determining the existence of feedback mechanisms between progenitors at different stages of differentiation and the upstream more pluripotent progenitors is critical to understand how lineage differentiation is controlled at the organ level.

The niche has two different types of stem cells – the HSC and the MSC. Most likely, the relationship between these two types of stem cells is symbiotic. HSC and progenitors require MSC to self-renewal and/or differentiate. It is still unknown whether HSC and progenitors have an influence on MSC fate and whether MSC require HSC and progenitors to maintain their multipotent potential and avoid differentiation. In addition, the molecular mechanisms regulating the symbiotic relationship between HSC and MSC will be important players in designing therapeutic approaches to modulate hematopoiesis, in a far ahead future.

The understanding of niche regulation is not only important for immunology and/or development biology but also to biology in general because it will shed light on maintenance of tissue homeostasis.

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Appendix| Publications

1. Beck, TC, **Gomes, AC**, Cyster, JG, Pereira, JP. (2014) CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow. *J. Exp. Med.* 211 (13): 2567–258

2. **Gomes, AC***, Hara, T*, Lim, V, Herndler-Brandstetter, D, Nevius, E, Sugiyama, T, Tani-ichi, S, Schlenner, S, Richie, E, Rodewald, HR, Flavell, R, Nagasawa, T, Ikuta, K, Pereira, JP (2015) HSC stem cell niches control multipotent progenitor differentiation. Submission proof.

3. Nevius, E*, **Gomes, AC***, Pereira, JP (2015) Inflammatory cell migration in rheumatoid arthritis. *Clinical Reviews in Allergy and Immunology*. Acceptance proof and article.

4. **Gomes, AC**, Vilanova, M, Gomes, MS (2015) *Submitted*. Article

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CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow

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Leukocyte residence in lymphoid organs is controlled by a balance between retention and egress-promoting chemoattractants sensed by pertussis toxin (PTX)–sensitive Gαi protein–coupled receptors (GPCRs). Here, we use two-photon intravital microscopy to show that immature B cell retention within bone marrow (BM) was strictly dependent on amoeboid motility mediated by CXCR4 and CXCL12 and by α4β1 integrin–mediated adhesion to VCAM-1. However, B lineage cell egress from BM is independent of PTX-sensitive GPCR signaling. B lineage cells expressing PTX rapidly exited BM even though their motility within BM parenchyma was significantly reduced. Our experiments reveal that when immature B cells are near BM sinusoids their motility is reduced, their morphology is predominantly rounded, and cells reverse transmigrate across sinusoidal endothelium in a largely non-amoeboid manner. Immature B cell egress from BM was dependent on a twofold CXCR4 down-regulation that was antagonized by antigen-induced BCR signaling. This passive mode of cell egress from BM also contributes significantly to the export of other hematopoietic cells, including granulocytes, monocytes, and NK cells, and is reminiscent of erythrocyte egress.

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Abbreviations used: GPCR, Gαi protein–coupled receptor; HEL, hen egg lysozyme; IVM, intravital microscopy; PB, peripheral blood; PTX, pertussis toxin.

Leukocyte egress from lymphoid organs is a multistep process characterized by active cell migration mediated by pertussis toxin (PTX)–sensitive Gαi protein–coupled receptors (GPCRs) toward exit sites, followed by reverse transmigration across endothelial barriers. Lymphocyte egress from thymus and lymph nodes is highly dependent on the chemoattractant lipid sphingosine 1 phosphate (S1P), which is abundant in circulatory fluids (blood and lymph) while limited in the lymphoid organ interstitium. The S1P gradient is sensed by lymphocytes through intrinsic expression of the PTX-sensitive GPCR S1P receptor 1 ($S1PR_1$). $S1PR_1$ deficiency causes ∼50–1,000-fold reduction in T and B lymphocyte numbers in blood and lymph concomitant with their significant accumulation in lymphoid organs (Cyster and Schwab, 2012). $S1PR_1$ mRNA expression is driven by the transcription factor Krüppel-like factor-2 (KLF2) in developing thymocytes and in naive T lymphocytes (Carlson et al., 2006; Bai et al., 2007). Of note, KLF2 transcription is dependent on the FOXO1 transcription factor (Fabre et al., 2008; Gubbels Bupp et al., 2009; Kerdiles et al., 2009), and in T cells FOXO1 is sequestered in the cytoplasm and rendered transcriptionally inactive via phosphorylation mediated by the serine/threonine kinase AKT (Fabre et al., 2005). This molecular circuitry seems to ensure that only the negatively selected thymocytes undergoing low TCR signaling achieve sufficient $S1PR_1$ expression for exiting the thymus. In contrast, S1P and its receptors play a modest role in mediating cell egress from BM, as genetic or pharmacologically induced S1P receptor deficiency only accounts for approximately two- to threefold reduction in immature B lymphocyte, NK cell, and eosinophil export from BM (Walzer et al., 2007; Jenne et al., 2009; Allende et al., 2010; Pereira et al., 2010; Sugita et al., 2010). $S1PR_1$ mRNA expression is largely independent of KLF2 expressed in developing and mature B lymphocytes (Hart et al., 2011), thus making it unlikely that the S1P/$S1PR_1$ egress pathway is under the

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control of BCR signaling induced in immature B lymphocytes during negative selection in BM. The mechanism or mechanisms used by immature B lymphocytes for exiting BM thus remain essentially unknown.

Whereas T cells comprise the vast majority of cells exported from the thymus, all other hematopoietic cells, and several nonhematopoietic cells, are produced in and exported from the BM. Neutrophils and monocytes use the GPCRs CXCR2 and CCR2 for BM egress, respectively; however, deficiency in either receptor reduced BM export by less than sevenfold (Serbina and Pamer, 2006; Eash et al., 2010; Shi et al., 2011). Why are lymphocytes highly sensitive to $S1PR_1$-dependent mechanisms for exiting thymus and lymph nodes, whereas other hematopoietic cells, including lymphocytes, are marginally dependent on single GPCR-dependent mechanisms for egress from BM? One possibility is that redundancy with multiple GPCRs controls egress of different cell lineages from BM. Alternatively, the fact that millions of red blood cells are produced and exported daily from BM (Lichtman and Santillo, 1986), and that these cells lack mechanisms for interstitial amoeboid cell migration, raises the possibility that alternative mechanisms control hematopoietic cell egress from BM.

CXCR4 is a PTX-sensitive GPCR that signals the BM homing and retention of multiple hematopoietic cell lineages, including hematopoietic stem and progenitor cells, monocytes, neutrophils, NK cells, B cells, and plasma cells (Ma et al., 1999; Hargreaves et al., 2001; Lapidot and Kollet, 2002; Liles et al., 2003; Broxmeyer et al., 2005; Bernardini et al., 2008; Pereira et al., 2009; Wang et al., 2009b; Eash et al., 2010). CXCL12, the CXCR4 ligand, is a potent chemoattractant to various hematopoietic cells and is abundantly expressed by stromal cells, osteoblasts, and endothelial and perivascular cells in BM (Sugiyama et al., 2006; Ding and Morrison, 2013). CXCR4/CXCL12 counteracts the activity of egress-promoting cues in immature B cells, neutrophils, NK cells, and monocytes (Bernardini et al., 2008; Wang et al., 2009b; Allende et al., 2010; Eash et al., 2010), though how CXCR4 signaling antagonizes cell egress remains unknown.

In this study, we demonstrate that CXCR4 signaling controls B lineage cell motility within BM parenchyma. Furthermore, developing B cell migration is strictly dependent on α4β1 integrin–mediated adhesion to VCAM-1. The BM parenchyma is extensively perfused by blood flow, which presumably imposes significant shear stress on BM-resident cells. Deficiency in CXCR4-mediated B lineage cell motility in BM parenchyma resulted in their acute mobilization from BM into periphery. Likewise, B lineage cells expressing PTX are predominantly nonmotile in BM parenchyma and are efficiently mobilized into the periphery. Our experiments revealed that the critical mechanism regulating B lineage cell egress from BM is a twofold down-regulation of CXCR4 in immature B cells, which depolarizes their amoeboid shape, reduces cell movement in perisinusoidal niches, and facilitates export into BM sinusoids and peripheral organs. Notably, antigen engagement by immature B cells prevents CXCR4 down-regulation, increases B lineage cell motility in BM parenchyma, and blocks egress from BM.

RESULTS

CXCR4 controls B lineage cell movement within BM

We examined how CXCR4 inhibits BM egress by visualizing the dynamic behavior of developing B lymphocytes within calvarial BM of *Rag1*$^{GFP/+}$ mice by two-photon intravital microscopy (IVM) before and after disruption of CXCR4 signaling. Mice were injected i.v. with fluorescently labeled dextrans to allow distinguishing BM parenchyma from sinusoids. Approximately 99.8% of GFP$^+$ cells in BM are B lineage cells (99.5% B220$^+$ cells and 0.3% B220^{lo} CD93$^+$ early B lineage progenitors), ∼0.1% are CD3e$^+$ T cells (presumably recent thymic emigrants), and CD3e$^-$ NK1.1$^+$ cells were all found to be GFP$^-$ (not depicted). GFP$^+$ B lineage cells displaced throughout the 30-min imaging period (Fig. 1, A and B) at a median velocity of 2.4 µm/min (Fig. 1 C), as expected (Pereira et al., 2009). However, cells rapidly stopped their migration 1–2 min after treatment with 80 µg of the CXCR4 antagonists AMD3100 or TN14003 (Tamamura et al., 2001) i.v. or 1 h after treatment with CXCL12 blocking antibody (Fig. 1, A and B; and Video 1). The sudden arrest led to a significant reduction in the median velocity (Fig. 1 C) and mean displacement (Fig. 1 D), which were reflected in a 6–10-fold reduction of the mean motility coefficient (Fig. 1 B). CXCR4 signaling blockade also changed B cell morphology from an amoeboid/elongated form to a significantly rounded shape (Fig. 1, E and F; and Video 1). The effects of AMD3100 were reversible after 2–3 h, with most cells regaining an amoeboid shape and motility (not depicted), which is in agreement with its rapid decay rate in vivo (Hendrix et al., 2000). These data suggested an intrinsic requirement for CXCR4 for B cell migration in vivo. Indeed, B cells conditionally deficient in CXCR4 moved within BM with a significantly reduced median velocity (Fig. 1 H and Video 2) and were more rounded than WT B cells (Fig. 1 I). However, most cells were not entirely stopped (Video 2), and their mean motility coefficient was only reduced by ∼40% (Fig. 1 G). Treatment with AMD3100 (80 µg i.v.; Video 3) further reduced the velocity and mean motility coefficient of CXCR4-deficient B cells and increased their roundness (Fig. 1, G–I). The similar activities of the three CXCR4 signaling inhibitors reduced the probability that B cell motility defects were caused by off-target effects. Also, in vitro B cell migration toward the chemokine CXCL13 was unaffected by treatment with AMD3100 (not depicted). Genetic and pharmacological CXCR4 deficiency also led to the significant mobilization of developing B cell subsets from BM into blood (Fig. 1 J), as expected (Nie et al., 2004; Pereira et al., 2009). As CXCR4 is critically required for B lineage cell retention in BM, it is possible that the few GFP$^+$ B lineage cells observed in calvaria BM of CXCR4 conditionally deficient mice had residual CXCR4 signaling that was sufficient for promoting BM retention and migration. Indeed, some CXCR4 conditionally deficient B lineage cells exhibited low CXCR4 surface expression (not depicted). Nevertheless, these data do not exclude the possibility that lymphocyte-extrinsic CXCR4 contributes to B lineage cell motility in BM.

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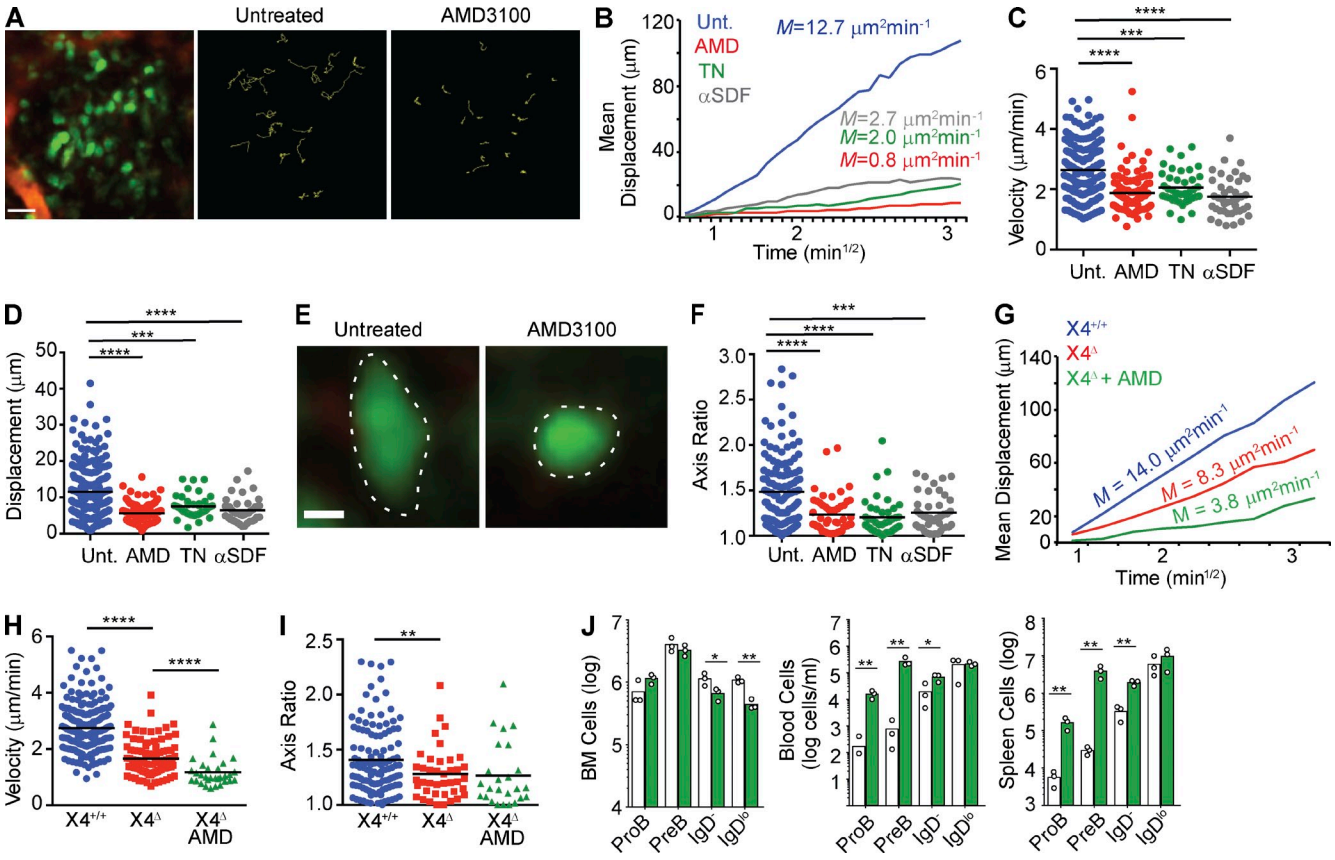

Figure 1. CXCR4 antagonism inhibits B lineage cell migration in BM. (A–F) *Rag1*$^{GFP/+}$ mice were injected i.v. with AMD3100, TN14003, or anti-CXCL12 antibody. Blood vessels were labeled with 2,000-kD dextran-rhodamine injected i.v. Cell movement was tracked by IVM of calvaria immediately before and after treatment. (A, left) Distribution of GFP$^+$ B cells (green) in BM. (middle and right) Movement of GFP$^+$ B cells tracked for 30 min before and after AMD3100 treatment, respectively. Colored lines represent cell trajectories. Data are representative of three individual experiments. (B) Mean motility coefficient of B lineage cells before (blue) and after (red) treatment with CXCR4 antagonists. Cell displacement from starting coordinates is plotted against the square root of time. Lines depict the average mean motility coefficient calculated from three individual experiments. (C) Median velocity (μm/min). (D) Displacement (μm). (E) Morphology of GFP$^+$ B lineage cells before (left) and after (right) AMD3100 treatment. Dotted lines depict the cells' borders. Bars: (A) 20 μm; (E) 30 μm. (F) Measurement of cell axis ratio of GFP$^+$ B lineage cells before (blue) and after (red) treatment with CXCR4 inhibitors. (G–I) WT (*Mb1*$^{Cre/+}$) and CXCR4 KO (*Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/-}$) were treated as in A–F. (G) Mean motility coefficient of WT (blue) and CXCR4 KO (red) B lineage cells before (red) and after (green) treatment with AMD3100. Colored lines depict average mean motility coefficient calculated from three independent datasets. (H) Median cell velocity of WT (blue) and CXCR4 KO (red) B lineage cells before (red) and after (green) treatment with AMD3100. (I) Cell axis ratio of GFP$^+$ (*Mb1*$^{Cre/+}$, blue) and CXCR4 KO (*Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/-}$, red) B lineage cells. (G–I) Data are representative of two independent experiments. (C, D, F, H, and I) Lines indicate mean. (J) Enumeration of B lineage cells in BM, blood, and spleen of *Mb1*$^{Cre/+}$ *Cxcr4*$^{+/+}$ (open bars) and *Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/-}$ (green bars) mice. Bars indicate mean, and circles indicate individual mice. Data are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$ by unpaired Student's *t* test.

Integrin-dependent B lineage cell migration in BM

Leukocyte migration is independent of integrin-mediated adhesion within lymphoid organ interstitium (Woolf et al., 2007; Lämmermann et al., 2008). In contrast, leukocytes require integrins for migration on two-dimensional surfaces (haptokinetic migration), such as migration along luminal endothelium toward the site of diapedesis (Alon and Feigelson, 2009). The cell shape changes that occurred immediately after treatment with CXCR4 inhibitors suggested that CXCR4 was involved in promoting B cell adhesion to the BM extracellular matrix and raised the possibility that B lymphocyte migration within BM interstitium was haptokinetic (i.e., integrin dependent). Consistent with this possibility, CXCR4 signaling increases α4β1 integrin affinity for its ligand VCAM-1, and both contribute to B lineage cell retention in BM (Koni et al., 2001; Leuker et al., 2001; Glodek et al., 2003; Pereira et al., 2009). To test this hypothesis, we analyzed B cell motility within BM parenchyma of *Rag1*$^{GFP/+}$ mice by IVM before and after i.v. administration of 200 μg anti-α4 (clone PS/2) or anti–VCAM-1 (clone M/K-2) blocking antibodies. B cell migration was largely blocked after treatment with anti-α4 or anti–VCAM-1 antibodies (Fig. 2 A and Video 4). There was a sixfold reduction of the mean motility coefficient (Fig. 2 B), reduced cell velocity (Fig. 2 C), and increased cell roundness (Fig. 2 D). The requirement for α4β1 integrin–mediated haptokinesis was intrinsic to B lineage cells as similar

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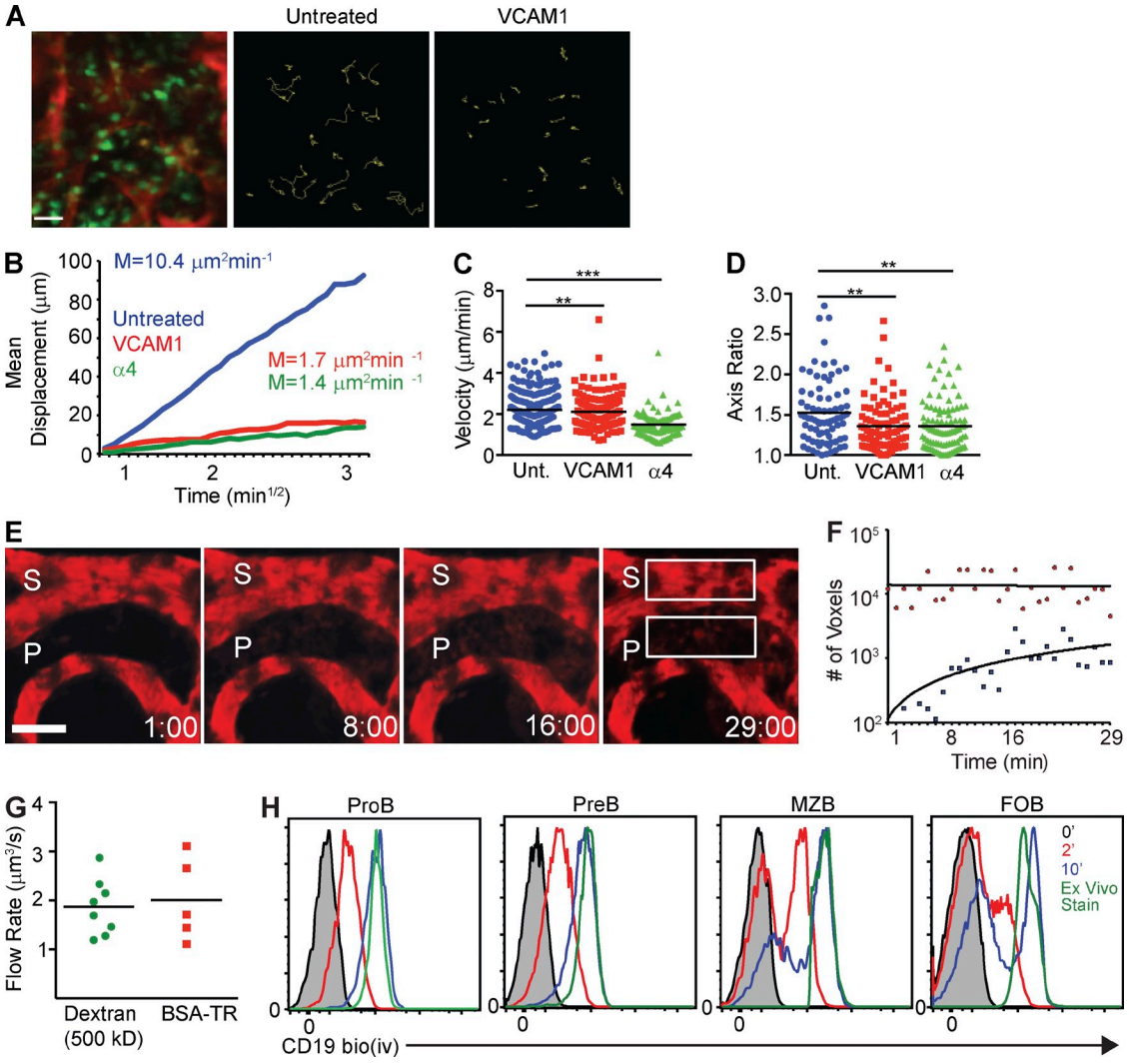

Figure 2. Developing B cell motility in BM parenchyma is strictly dependent on α4β1 integrin–mediated adhesion to VCAM-1. (A–D) *Rag1*$^{GFP/+}$ were injected (i.v.) with integrin α4 or VCAM-1 blocking antibodies, and calvarial BM was imaged before and immediately after treatment. Blood vessels were labeled with 2,000-kD dextran-rhodamine injected i.v. (A, left) Distribution of GFP$^+$ B cells (green) in BM. (middle and right) Movement of GFP$^+$ B cells tracked for 30 min before and after treatment with anti–VCAM-1 antibody. Colored lines represent cell trajectories. (B) Mean motility coefficient of B lineage cells before (blue line) and after VCAM-1 (red) and α4 blockade (green). Lines depict the average mean motility coefficient calculated from three independent experiments. (C) Median velocity (μm/min). (D) Cell axis ratios before (blue) and after treatment with VCAM-1 (red) and α4 (green) blocking antibodies. The data are representative of three independent experiments. (C and D) Lines indicate mean. (E) Mice were injected with 50 μg of 500-kD FITC-conjugated dextran (i.v.), and calvaria BM was imaged before and after treatment. BM vasculature (S) and parenchyma (P) are indicated. White boxes delineate regions of interest used for measurements of dextran perfusion. Time is shown in each panel (mm:ss). (A and E) Bars, 20 μm. (F) FITC-conjugated dextran perfusion of BM parenchyma and vasculature (red fluorescent voxels; y axis) over 30 min (time; x axis). Data are representative of >10 independent experiments. (G) Interstitial fluid flow rate (μm^3/s) determined after injection (i.v.) of FITC-dextran (500 kD; green) or BSA–Texas red (red). Lines indicate the mean; symbols represent regions of interest obtained from three mice (FITC-dextran) or from two mice (BSA–Texas red). (H) In vivo staining of pro-B and pre-B cells in BM and of marginal zone (MZB) and follicular (FOB) B cell subsets in spleen with biotin–anti-CD19 antibody administered i.v. for 2 (red) or 10 (blue) min. Saturation with anti-CD19 antibody was determined by ex vivo staining with excess antibody (green). Data are representative of three independent experiments. **, $P < 0.005$; ***, $P < 0.0005$ by unpaired Student's *t* test.

observations were made in *Itgb1*-deficient B lymphocytes (Video 5). Treatment with anti-α4 blocking antibody did not change the dynamic behavior of *Itgb1*-deficient B cells (not depicted). Interestingly, loosely adherent and rounded cells were seen occasionally moving short distances for a brief period (Video 6). The number of developing B cells was significantly reduced in BM, which corresponded to a significant increase in peripheral blood (PB; not depicted), as expected (Pereira et al., 2009). Electron microscopy analyzes of the BM sinusoidal endothelium demonstrated that it is highly fenestrated (Tavassoli and Yoffey, 1983). Consistent with these observations, the BM vasculature was permeable to large-molecular-mass, FITC-coupled

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dextrans (~500 kD) present in circulatory fluids (Fig. 2, E and F; and Video 7), and blood plasma perfusion reached an interstitial fluid flow rate of ~2 $\mu m^3/s$ (Fig. 2 G). The extensive perfusion of the BM parenchyma within minutes of injection of intravascular dyes is consistent with BM-resident cells being continuously exposed to shear stress generated by fluid flow. Indeed, considering that the femur volume is <100 μm^3 (our unpublished estimates), we hypothesized that circulatory molecules access the entire femur BM parenchyma in <2 min. To test for this possibility, we injected 5 µg biotin-conjugated anti-CD19 antibody i.v., allowed it to equilibrate for 2 or 10 min, and measured the extent of binding to pro-B and pre-B cells in BM. For comparison we examined labeling of splenic follicular and marginal zone B cells of the same mouse. Our data show that the large majority of pro-B and pre-B cells are readily labeled with anti-CD19 within 2 min, and by 10 min 100% of each B cell subset is saturated with circulatory antibody (Fig. 2 H). In contrast, a significant fraction of mature follicular B cells in the spleen is poorly stained even after 10 min of exposure to biotin anti-CD19 antibody in circulation. As expected, the marginal zone B cell subset was readily stained with anti-CD19 antibodies after 2 min (Cinamon et al., 2008), but saturation was only achieved after 10 min (Fig. 2 H). These data demonstrate that circulatory molecules rapidly perfuse BM parenchyma. Whether lymphocytes access BM exit sites by forces induced upon cells, such as interstitial fluid flow, or exclusively by active migration guided by chemoattractants sensed by GPCRs intrinsically expressed on lymphocytes was not clear.

B lineage cell egress from BM is independent of GPCR-guided amoeboid motility

The findings that CXCR4 blockade significantly reduced B lineage cell motility in parenchyma while accelerating egress into sinusoids and PB raised an interesting and unprecedented possibility: that BM egress could be achieved in the absence of amoeboid migration toward BM exit sites. To test this hypothesis, we conditionally induced PTX expression in B lineage cells by crossing $Mb1^{Cre/+}$ mice with mice encoding the ADP-ribosyl transferase S1 subunit of PTX within the *Rosa26* locus, preceded by a premature transcriptional stop codon flanked by loxP sites (for simplicity referred as $Rosa26^{PTX/+}$ mice; Regard et al., 2007). Using this strategy, >99% of B lineage cells conditionally express genes under *Rosa26* promoters from the pro-B cell stage and throughout subsequent stages of development (not depicted). The number of pro-B and pre-B cells was slightly but significantly reduced in BM, which corresponded to a small, but highly significant numerical increase in blood circulation (Fig. 3, A and B). However, IgM^+ immature B cell subsets were reduced by two- to threefold in BM and significantly increased in blood, indicating increased BM egress. We did not find evidence of bystander effects, such as PTX leakiness into neighboring cells in BM mixed chimeras (not depicted). Furthermore, similar findings were obtained with B lineage cells deficient in *Gnai2* and *Gnai3* (Hwang et al., 2013). PTX-expressing B lineage cells were also poorly retained within BM sinusoids (not depicted), consistent with a role for Gαi-coupled CB2 and S1PR3 in this process (Pereira et al., 2009; Donovan et al., 2010). Mature B lymphocytes were also reduced in BM (Fig. 3 A) because CXCR4 coupling to Gαi proteins is PTX sensitive, and mature B cells require CXCR4 for homing back to BM (Nie et al., 2004; Pereira et al., 2009).

To investigate whether B lineage cell motility within BM was dependent on PTX-sensitive GPCRs, we visualized the behavior of PTX-expressing B cells marked with a $Rag1^{GFP/+}$ allele in calvaria BM by IVM. We found that PTX-expressing B cells were predominantly nonmotile (Fig. 3, C and D; and Video 8) and exhibited rounded morphology (Fig. 3 E). Occasionally, we found BM pockets with some PTX-expressing B cells moving in an amoeboid manner with similar kinetics as WT cells (Video 9), but their migration was sensitive to CXCL12 blocking antibody treatment (Fig. 3 F). These findings suggest that CXCR4 can couple to PTX-insensitive G proteins in B cells and promote interstitial migration in vivo. In agreement with these observations, AMD3100 blocked the chemotaxis of WT and PTX-expressing developing B cell subsets toward a CXCL12 gradient in vitro, whereas CXCR4-deficient B cells were unable to migrate in similar conditions (not depicted). Finally, we asked whether other hematopoietic cells could leave BM independently of PTX-sensitive GPCRs. To address this question, we treated mice with 1 µg PTX i.v. for 24 h and quantified hematopoietic cell egress from BM into PB by flow cytometry. NK cells, neutrophils, and inflammatory monocytes, like B lineage cells, were efficiently mobilized from BM into PB by PTX treatment (Fig. 3 G), even though PTX-sensitive GPCRs can contribute to their export from BM (Serbina and Pamer, 2006; Walzer et al., 2007; Jenne et al., 2009; Allende et al., 2010; Eash et al., 2010; Pereira et al., 2010). Similar results were obtained by cre recombinase–mediated PTX expression in granulocytes and monocytes and in NK cells, driven by *Lyz2* (Clausen et al., 1999) or by *Il7r* (Schlenner et al., 2010), respectively (not depicted).

The increased rate of BM B cell egress seen in CXCR4-deficient mice, or in PTX-expressing B cells, suggested that defective motility within parenchyma enforced B lineage cells to position near BM sinusoids. To address this possibility, we measured the distance between WT, CXCR4-deficient, and PTX-expressing B lineage cells and BM sinusoids and found that both CXCR4-deficient and PTX-expressing cells were significantly accumulated in proximity to sinusoids (perisinusoidal space was defined as the area that is <10 µm distal from sinusoids; Fig. 4, A and B). As CXCR4 controls the movement of B lineage cells in parenchyma and the lack of CXCR4 signaling positions cells around sinusoids (Fig. 4, A and B) and within sinusoids (Pereira et al., 2009), we hypothesized that B lineage cells moving within perisinusoidal niches had reduced motility. Thus, we analyzed the dynamic behavior of B lineage cells that moved within 10-µm distance to sinusoids and compared them with cells moving in parenchymal areas of the same imaging volume. Even though there was a trend toward reduced median velocity that did not reach statistical significance

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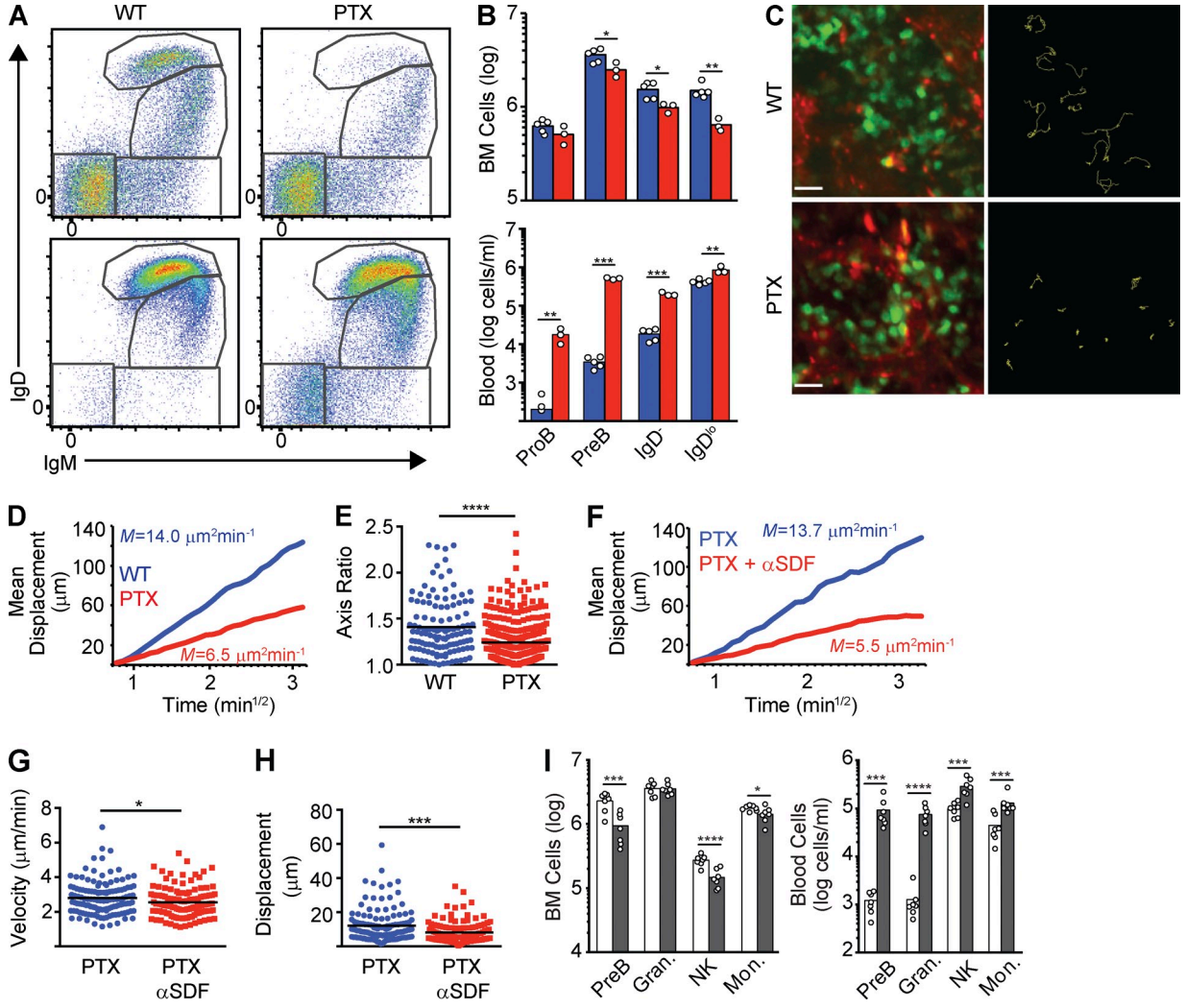

Figure 3. B cells egress BM independently of GPCR-mediated migration. (A) IgM and IgD expression in live gated (DAPI$^-$) B220$^+$ cells in BM (top) and PB (bottom) of *Mb1*$^{Cre/+}$ (WT, left) and *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice (PTX, right). (B) Quantification of developing B cell subsets in BM (top) and PB (bottom) of *Mb1*$^{Cre/+}$ mice (blue bars) and *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice (red bars). Bars indicate mean, and circles depict individual mice analyzed. Data are representative of more than three independent experiments. (C) IVM of PTX-expressing B lineage cells in calvaria BM. Blood vessels were visualized with BSA–Texas red. (top) Distribution of GFP$^+$ B cells (left) and the movement of GFP$^+$ B cells tracked for 30 min in BM (right) of *Mb1*$^{Cre/+}$ mice (WT). (bottom) Distribution of GFP$^+$ B cells (left) and the movement of GFP$^+$ B cells tracked for 30 min in BM (right) of *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice (PTX). Colored lines represent cell trajectories. The data are representative of at least three independent experiments. Bars, 20 µm. (D) Mean motility coefficient of WT and PTX-expressing B lineage cells. Lines depict the average mean motility coefficient calculated from three independent mice. (E) Cell axis ratios of WT and PTX-expressing cells. (F–H) Analysis of the mean motility coefficient (F), median velocity (µm/min; G), and displacement (µm; H) of PTX-expressing B lineage cells before (blue) and after treatment with 250 µg anti-CXCL12 antibody (αSDF, red). M indicates mean motility coefficient in µm^2/min. Lines depict the average mean motility coefficient calculated from datasets obtained by IVM of three different mice. (E, G, and H) Lines indicate mean. (I) Numbers of pre-B cells, NK cells, granulocytes (Gran.) and inflammatory monocytes (Mon.) in BM and PB of WT mice treated with saline (open bars) or with 1 µg PTX for 24 h (gray bars). Data are representative of more than three independent experiments. *, $P < 0.05$; **, $P < 0.005$, ***, $P < 0.0005$; ****, $P < 0.00005$ by unpaired Student's *t* test.

(Fig. 4 C), their motility coefficient was reduced by 35% (Fig. 4 D), and cells within perisinusoidal niches were significantly less amoeboid (Fig. 4 E). B cells were only rarely observed migrating across sinusoidal endothelium before or after CXCR4 blockade, or in B cells genetically deficient in CXCR4 or in Gαi protein–dependent receptors. This was not surprising because the numerical increase of Gαi protein– or CXCR4-deficient B cell subsets in blood is ∼10–100-fold lower than the total number of B lineage cells remaining in BM (Fig. 3, A and B; and not depicted, respectively). Furthermore, the relatively small number of B lineage cells visualized and the limited volume of imaging fields make it difficult to capture B cells transmigrating across sinusoidal endothelial barriers. However, in ∼18 h of IVM of untreated WT (*Rag1*$^{GFP/+}$) mice and in 3 h of IVM of PTX-expressing B cells, we were able to observe reverse transmigration of 11 and 2 B lineage cells,

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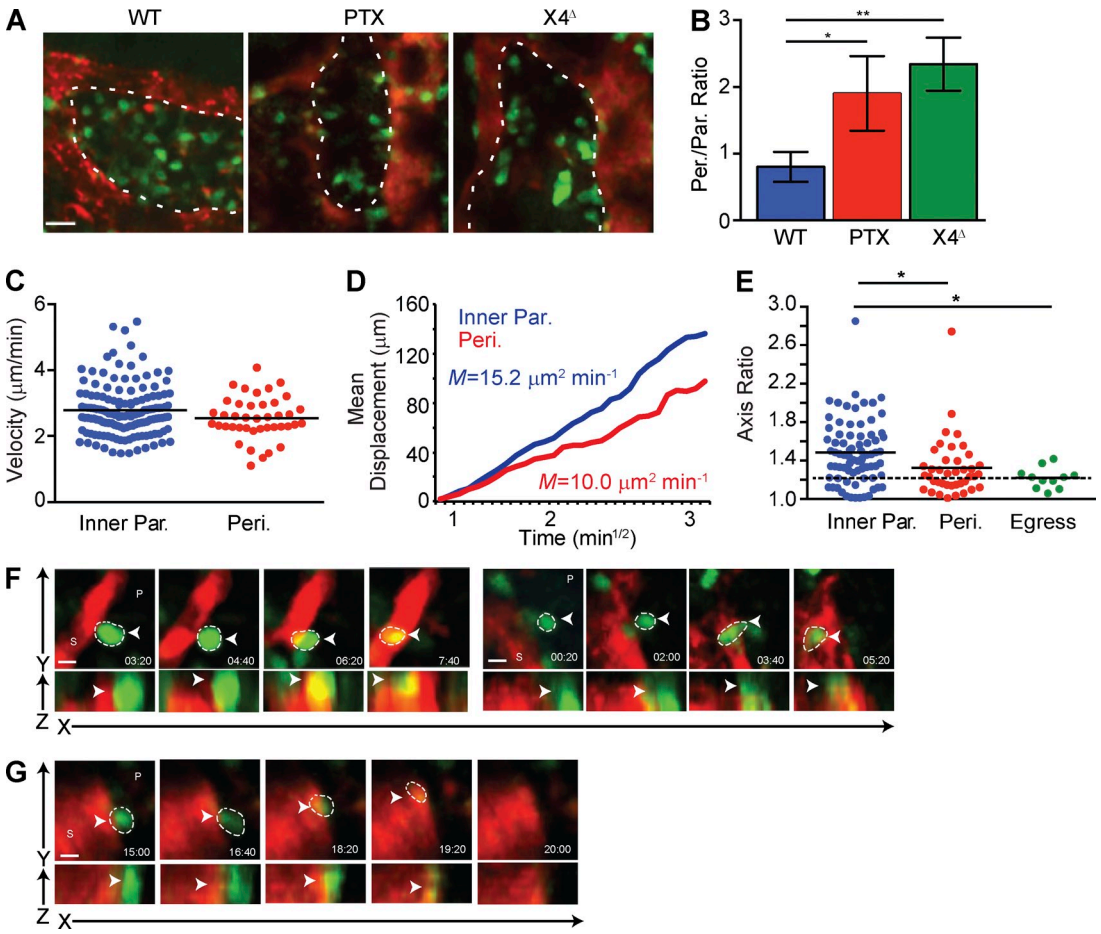

Figure 4. Morphology and motility of developing B cells during BM egress. (A) Distribution of B lineage (*Rag1*$^{GFP/+}$) cells in BM of *Mb1*$^{Cre/+}$ *Rosa26*$^{+/+}$ mice (WT, left), *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ mice (PTX, middle), and *Mb1*$^{Cre/+}$ *Cxcr4*$^{fl/-}$ mice (X4$^{\Delta}$, right). Dotted lines indicate border between sinusoids and parenchyma. (B) Ratio of B lineage cells proximal (<10 µm) and distal (>10 µm) to BM sinusoids. Data depicted were from >90 B lineage cells analyzed in three WT, PTX, and X4$^{\Delta}$ mice. Bars indicate mean (±SEM). (C–E) Cell motility parameters of developing B cells in parenchyma and in perisinusoidal space. (C) Median velocity (µm/min). (D) Mean motility coefficient of *Rag1*$^{GFP/+}$ B lineage cells in inner parenchyma (Inner Par. blue) and in perisinusoidal areas (Per. red). Mean cell displacement from starting coordinates is plotted against the square root of time. Lines depict the average mean motility coefficient of >70 inner parenchyma and 40 perisinusoidal areas GFP^{+} B cells. (E) Axis ratio (x/y). Dotted line depicts the mean axis ratio of *Mb1*$^{Cre/+}$ *Rosa26*$^{PTX/+}$ B cells in BM parenchyma (1.2). (C and E) Lines indicate means. (F and G) Two WT GFP^{+} B cells (F) and one PTX-expressing GFP^{+} B cell (G) exiting from BM parenchyma (P) into sinusoids (S). Arrowheads point to cell egress. Time is shown in mm:ss. WT and PTX-expressing cell egress examples were captured from more than 10 and 4 independent experiments, respectively. Bars: (A) 19 µm; (F and G) 5 µm. *, $P < 0.05$; **, $P < 0.005$ by unpaired Student's *t* test. Unpaired Student's *t* test with Welch's correction was used in E.

respectively. In all examples observed, B lineage cells that exited BM parenchyma into sinusoids did not approach BM exit sites in an amoeboid manner, were poorly motile, and resided adjacent to sinusoids (Video 10). Consistent with our previous findings, we did not observe morphological differences between WT and PTX-expressing B cells in the process of exiting into sinusoids (Fig. 4, F and G; and Video 10), even though PTX-expressing B cells were rapidly displaced into circulation after entry into sinusoids, whereas WT B lineage cells were retained in this compartment, as expected (Pereira et al., 2009; Donovan et al., 2010). Interestingly, reverse transmigration occurred exclusively in areas where the sinusoidal endothelial barrier exhibited intense vascular leakage (Video 10). In some cases, WT and PTX-expressing cells deformed during reverse transmigration, which strongly suggests that reverse transmigration across BM sinusoidal endothelium is an active process that is independent of GPCR signaling.

Gradual reduction in CXCR4 expression reduces motility and enforces immature B cell egress from BM

Previous studies have shown that CXCR4 expression is highest at the pro-B cell stage and decreases gradually during murine and human B cell development (Honczarenko et al., 1999; Pereira et al., 2009). The finding that perisinusoidal B lineage cells exhibited reduced motility and increased roundness suggested that CXCR4 signaling was reduced in egress-competent cells. To investigate this possibility, we compared CXCR4 expression on immature B lymphocyte subsets before and after

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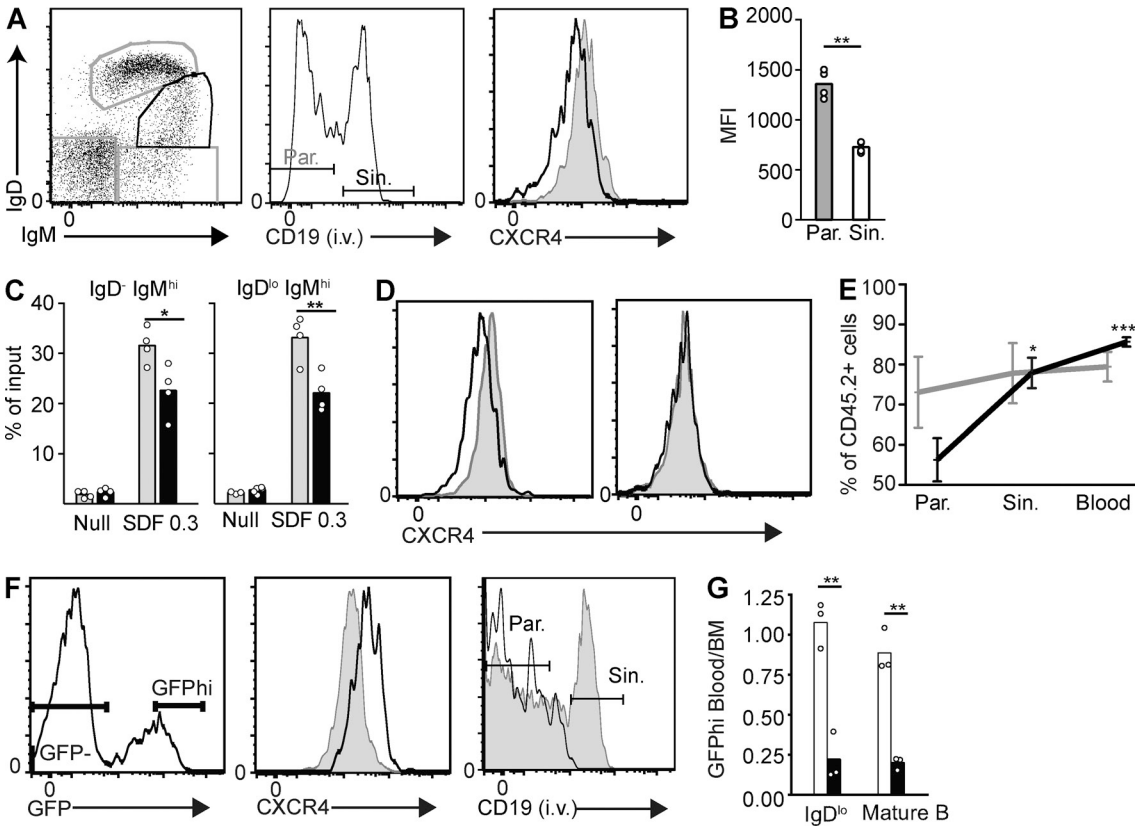

Figure 5. CXCR4 expression in egress-competent immature B lymphocytes. (A) CXCR4 expression in B220$^+$ immature IgM$^+$ IgD^{lo} B cells positioned in BM parenchyma (Par.) and sinusoids (Sin.). (left) IgM and IgD expression in B220$^+$ BM cells. (middle) Distribution of B220$^+$ CD93$^+$ IgM$^+$ IgD^{lo} B cells in BM parenchyma (CD19-PE$^-$) and sinusoids (CD19-PE$^+$). (right) CXCR4 expression in B220$^+$ immature IgM$^+$ IgD^{lo} B cells in parenchyma (gray) and sinusoids (black line). (B) Geometric mean fluorescence intensity (MFI) of CXCR4 surface expression in BM parenchyma and sinusoid immature CD93$^+$ IgM$^+$ IgD^{lo} B cells from C57BL/6 mice ($n = 4$). Bars indicate the mean; circles indicate individual mice. Data are representative of >10 independent experiments. (C) Cell migration through 5-µm transwells. *Cxcr4*$^{+/+}$ (gray bars) and *Cxcr4*$^{+/-}$ (black bars) immature CD93$^+$ IgM^{hi} IgD$^-$ (left) and IgD^{lo} (right) cells were allowed to migrate toward 0.3 µg/ml CXCL12 for 3 h. Bars indicate mean, and circles indicate replicates. Data were pooled from two independent experiments. (D, left) CXCR4 surface expression in *Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/+}$ immature IgM$^+$ IgD^{lo} cells overlaid with *Mb1*$^{Cre/+}$ *Cxcr4*$^{+/+}$ immature IgM$^+$ IgD^{lo} cells in BM parenchyma. (right) CXCR4 surface expression in Ly5.1$^+$ *Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/+}$ immature IgM$^+$ IgD^{lo} cells in BM parenchyma overlaid with Ly5.2$^+$ (*Cxcr4*$^{+/+}$) immature IgM$^+$ IgD^{lo} cells residing in sinusoids. (E) Distribution of B220$^+$ CD93$^+$ IgM$^+$ IgD^{lo} immature B cells in BM parenchyma, sinusoids, and PB of lethally irradiated mice reconstituted with a mixture of Ly5.1$^+$ *Mb1*$^{Cre/+}$ *Cxcr4*$^{+/+}$ and Ly5.2$^+$ WT cells (gray) or with Ly5.1$^+$ *Mb1*$^{Cre/+}$ *Cxcr4*$^{Fl/+}$ and Ly5.2$^+$ WT cells (black). Error bars indicate ±SEM. (F) Overexpression of truncated CXCR4 (R334X WHIM mutation) in BM cell subsets reported by GFP expression. (left) GFP expression in gated B220$^+$ CD93$^+$ IgM$^+$ IgD^{lo} immature B cells. (middle) CXCR4 surface expression in gated GFP$^-$ (gray) and GFP$^+$ (black line) B220$^+$ CD93$^+$ IgM$^+$ IgD^{lo} immature B cells. (right) Distribution of CXCR4-transduced (GFP$^+$) B220$^+$ CD93$^+$ IgM$^+$ IgD^{lo} immature B cells in BM parenchyma and sinusoids. (G) Ratio of the frequencies of GFP^{hi} immature IgD^{lo} and mature B cells in PB and in BM. Bars indicate mean; circles indicate individual mice. Data were pooled from three independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by unpaired Student's *t* test.

BM egress and found that it was reduced by approximately twofold in cells within sinusoids as compared with cells in parenchyma (Fig. 5, A and B). This reduction was evident even after treatment with AMD3100 (not depicted), suggesting that it was not mediated by CXCR4 desensitization from exposure to perisinusoidal sources of CXCL12 (Ding and Morrison, 2013). A twofold reduction in CXCR4 was sufficient to reduce immature B lymphocyte migration toward a gradient of CXCL12 established in vitro (Fig. 5 C). To test whether a twofold down-regulation of CXCR4 was sufficient to mobilize immature B cells from BM, we compared the distribution of CXCR4 heterozygous B lineage cells in direct competition with CXCR4 WT cells in BM parenchyma, sinusoids, and PB. To accomplish this, we reconstituted lethally irradiated mice with a 50% mixture of BM cells isolated from *Cxcr4*$^{+/-}$ or *Cxcr4*$^{+/+}$ CD45.1 mice with *Cxcr4*$^{+/+}$ CD45.2 mice. As expected, *Cxcr4*$^{+/-}$ immature B cells in BM parenchyma expressed comparable CXCR4 surface amounts as *Cxcr4*$^{+/+}$ B cells positioned within BM sinusoids, whereas *Cxcr4*$^{+/-}$ immature B cells expressed approximately twofold lower CXCR4 than *Cxcr4*$^{+/+}$ immature B cells in BM parenchyma (Fig. 5 D). Furthermore, *Cxcr4*$^{+/-}$ immature B cells were significantly reduced in BM parenchyma and increased in BM sinusoids and blood when compared with *Cxcr4*$^{+/+}$ immature B cells (Fig. 5 E). Antagonizing CXCR4 down-regulation by retroviral transduction of hematopoietic stem cells with a desensitization-deficient

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CXCR4 (R334X mutation that causes the WHIM syndrome in humans [Hernandez et al., 2003]) was sufficient to prevent immature B lymphocyte egress from BM parenchyma into sinusoids and into PB (Fig. 5, F and G).

BCR signaling antagonizes CXCR4 down-regulation in immature B cells and prevents BM egress

In late stages of B cell development, newly generated immature B cells undergo negative selection against systemic self-antigens predominantly in BM (Goodnow et al., 2005). This process is likely promoted by immature B cell migration within BM by facilitating cellular interactions and screening for self-reactive BCRs. As B lineage cell migration in BM is controlled by CXCR4 and reduced CXCR4 signaling is essential for BM export, we hypothesized that BCR signaling intensity controlled CXCR4 expression in immature B cells. To test this hypothesis, we analyzed CXCR4 surface expression in immature B cells before and after inducing BCR stimulation. For this purpose, we isolated BM cells from WT mice (C57BL/6), MD4 BCR transgenic mice (B cells specifically recognize hen egg lysozyme [HEL]), and B1-8 BCR transgenic mice (nitrophenyl hapten–specific B cells) and induced BCR signaling with cognate antigens for various periods of time. We found that immature B cells increased CXCR4 surface expression as early as 3 h after treatment with stimulatory anti-IgM antibodies or with cognate antigens and peaked at 12 h after BCR stimulation (Fig. 6, A and B). We then asked whether self-antigen engagement was sufficient to prevent immature B cell egress from BM. We treated MD4 transgenic mice with 10 mg HEL for 6 h i.v. and measured immature B cell subsets in BM parenchyma, sinusoids, and PB. We found that HEL treatment not only significantly increased CXCR4 expression in immature B cells in vivo (Fig. 6 C), but also prevented immature B lymphocyte egress into sinusoids and PB by almost 10-fold (Fig. 6, D and E). The effects of HEL on CXCR4 expression and B cell egress blockade were strictly dependent on an HEL-specific BCR (Fig. 6 F). The peripheral reduction in immature B lymphocyte subsets was not caused by BCR signaling–induced cell death because these cells significantly accumulated in BM parenchyma (Fig. 6 E). Furthermore, similar results were obtained with MD4 immature B lymphocytes that overexpressed antiapoptotic BCL2 (Fig. 6 H). These results predicted that antigen engagement altered B lineage cell movement in BM parenchyma. To test this hypothesis, we crossed MD4 BCR transgenic mice with mice expressing cre recombinase driven by *Cd19* and with mice encoding the ZsGreen fluorescent protein driven by the chicken albumin and rabbit β globin promoter within the *Rosa26* locus, preceded by a premature transcriptional stop codon flanked by loxP sites (for simplicity referred as $Rosa26^{ZsGreen/+}$; Madisen et al., 2010). Using this strategy, ∼99% of $ZsGreen^+$ cells were IgM^+ B lineage cells (not depicted). We lethally irradiated C57BL/6 recipient mice and reconstituted them with BM cells taken from $MD4^+$ and $MD4^-$ $Cd19^{Cre/+}$ $Rosa26^{ZsGreen/+}$ mice. To measure the effect of antigen engagement mostly in immature B lymphocyte motility in BM, we analyzed the calvaria of BM chimeras by IVM 4 wk after reconstitution, a time point where ∼75% of $ZsGreen^+$ B lineage cells were immature B lymphocytes, as measured by CD93 surface expression (not depicted). In agreement with these findings, the amoeboid polarity and motility of $MD4^+$ B lymphocytes increased significantly after exposure to HEL, whereas the movement and morphology of $MD4^-$ B lineage cells was unaffected by HEL treatments (Fig. 6, I and J). We also noted that $MD4^+$ B cells were less motile and more rounded than $MD4^-$ B cells (Fig. 6, I and J). However, this difference was likely caused by the very limited size of pro-B and pre-B cell compartment in MD4 transgenic mice as compared with that in WT mice (not depicted). In summary, these data demonstrate that immature B cell motility and retention within BM parenchyma are significantly increased by BCR signaling.

DISCUSSION

Recent studies provided substantial mechanistic insight into the multistep process of lymphocyte egress from secondary lymphoid organs: as B and T lymphocytes randomly move via the activity of distinct chemoattractants, lymphocytes make frequent contacts and probe lymphoid organ sinuses. The availability of the egress-promoting chemoattractant S1P, presumably at sinusoidal exit sites, and signaling through the lymphocyte-intrinsic PTX-sensitive $S1PR_1$ are both required for lymphocyte reverse transmigration across sinusoidal endothelium and egress (Cyster and Schwab, 2012). Here, we demonstrate that developing B cell retention in BM parenchyma was dependent on lymphocyte-intrinsic amoeboid motility, predominantly mediated by CXCR4 signaling and by α4β1–VCAM-1. However, BM egress was surprisingly independent of egress-promoting chemoattractants sensed by PTX-sensitive GPCRs. PTX treatments in vivo have been shown to mobilize B cells and hematopoietic stem/progenitor cells from BM (Papayannopoulou et al., 2003; Ueda et al., 2004). In the work presented here, we now show that cell-intrinsic PTX expression not only reduces B lineage cell movement within BM parenchyma, but also mobilizes B lineage cells, NK cells, monocytes, and granulocytes from BM into the periphery.

The immature B cell stage is characterized by a significant change in positioning within BM, with up to 50% of immature B cells localizing within BM sinusoids in an α4β1–VCAM-1– and CB2 and $S1PR_3$ signaling–dependent manner (Pereira et al., 2009; Donovan et al., 2010). However, immature B cell movement toward sinusoids was independent of CB2- and $S1PR_3$-mediated chemotaxis and only partially contributed by S1P and $S1PR_1$ (Allende et al., 2010; Pereira et al., 2010). Our experiments now reveal that CXCR4 down-regulation enforced B cells to distribute into perisinusoidal and intrasinusoidal compartments and was critical for immature B cell egress from BM. Furthermore, these experiments also suggest that α4β1-mediated adhesion is temporally reduced when cells are in perisinusoidal compartments, before it is increased again within sinusoids, likely through CB2-induced transactivation (Pereira et al., 2009). It is possible that B cell–intrinsic α4β1 and VCAM-1 expressed on sinusoidal endothelial cells may antagonize reverse

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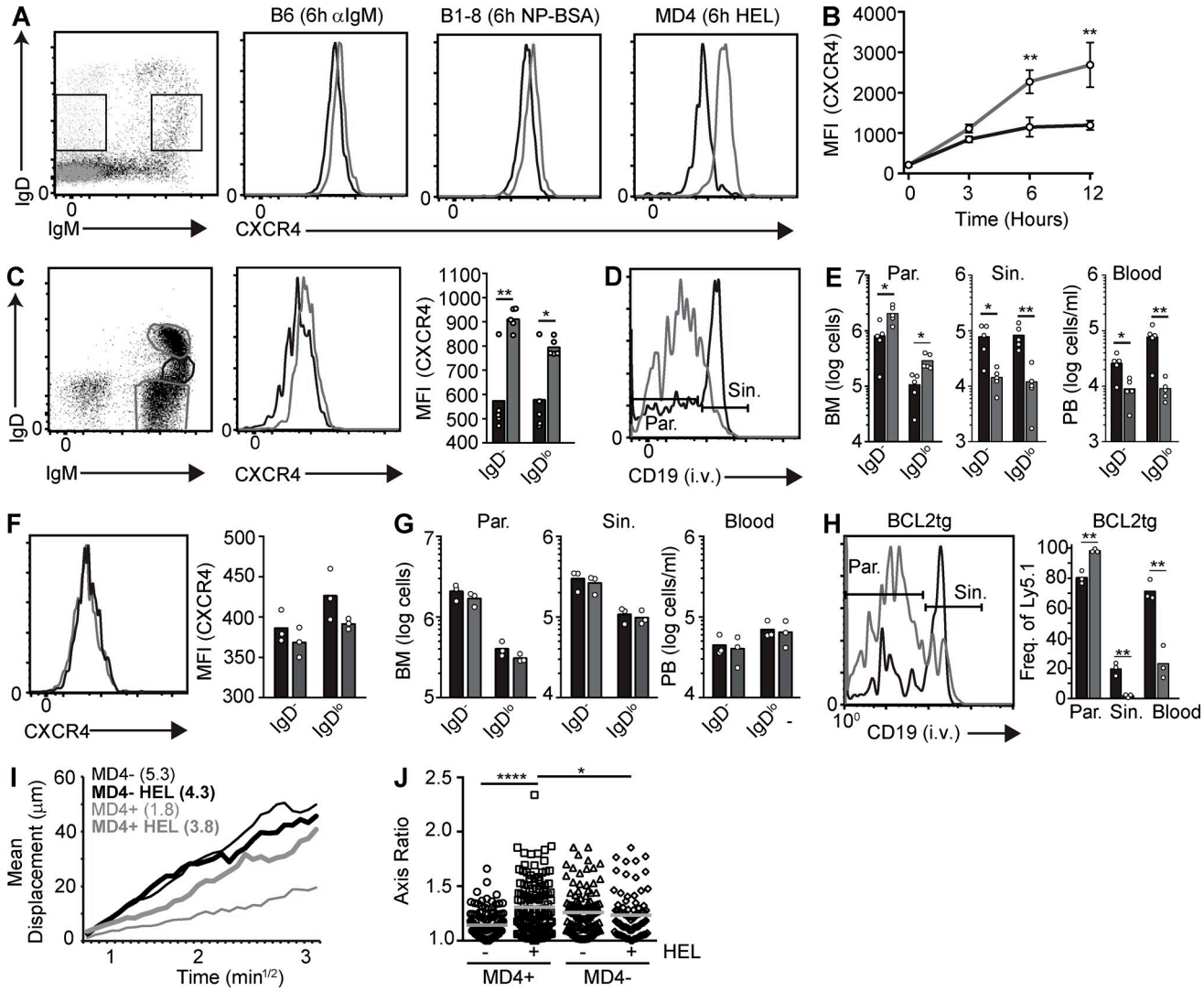

Figure 6. BCR signaling increases CXCR4 expression, promotes B lymphocyte motility, and prevents BM egress. (A) IgM and IgD expression in unstimulated and BCR-stimulated B220$^+$ cells (left and right gates, respectively). (B) CXCR4 mean fluorescence intensity (MFI) in IgM$^+$ IgD^{lo} CD93$^+$ MD4 B cells untreated (black) or stimulated with 1 mg/ml HEL (gray) for the indicated time periods. Lines indicate mean (±SEM) of three independent experiments. (C) CXCR4 surface expression in unstimulated (black) and in vivo HEL-stimulated (6 h; gray) immature (CD93$^+$) MD4 B cell subsets. (D) MD4 immature IgM$^+$ IgD^{lo} CD93$^+$ B cell distribution in BM before and after HEL i.v. treatment for 6 h. (E) Quantification of immature B cell subsets in BM parenchyma (Par.), sinusoids (Sin.), and PB of MD4 mice treated with HEL for 6 h. (F) CXCR4 surface expression in WT immature IgM$^+$ IgD^{lo} CD93$^+$ B cells stimulated with PBS (black) or with HEL i.v. (gray). (right) CXCR4 MFI on unstimulated (black) and HEL-stimulated (gray) immature IgM$^+$ IgD^{lo} CD93$^+$ B cells from WT mice. (G) Quantification of immature B cell subsets in BM parenchyma, sinusoids, and PB of WT mice treated with HEL for 6 h. (H, left) Distribution of immature IgM$^+$ IgD^{lo} CD93$^+$ MD4 B cells overexpressing Bcl2 in BM parenchyma (CD19-PE$^-$) and in sinusoids (CD19-PE$^+$). (right) Immature CD93$^+$ IgM$^+$ IgD^{lo} B cell numbers in BM parenchyma, sinusoids, and PB of MD4.BCL2 transgenic mice treated with HEL. (E, G, and H) Bars indicate mean, and circles depict individual mice. Data are representative of two independent experiments. (I) Mean motility coefficient of MD4$^+$ and MD4$^-$ *Cd19*$^{Cre/+}$ *Rosa26*$^{ZsGreen/+}$ B lineage cells before and 6 h after treatment with HEL i.v. Lines depict the average mean motility coefficient calculated from datasets obtained by IVM of two to three mice. (J) Axis ratio (x/y) of MD4$^+$ and MD4$^-$ *Cd19*$^{Cre/+}$ *Rosa26*$^{ZsGreen/+}$ B lineage cells before and 6 h after HEL treatment i.v. Circles, unstimulated MD4$^+$ B cells; squares, HEL-stimulated MD4$^+$ B cells; triangles, HEL-stimulated MD4$^-$ B cells; diamonds, MD4$^-$ unstimulated B cells. Lines indicate mean. Data are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.005$; ****, $P < 0.00005$ by unpaired Student's *t* test.

transmigration of developing B cell subsets and prevent their egress from BM. However, conditional deletion of VCAM-1 from endothelial (and hematopoietic) cells induced the selective mobilization of immature B cell subsets, most prominently immature IgM$^+$ IgD^{lo} cells (which are distributed between BM parenchyma and sinusoids), without significant effects measured in parenchyma-restricted pro-B and pre-B cell subsets (Koni et al., 2001). Thus, these data are in agreement with the finding that α4β1–VCAM-1 is specifically required for sinusoidal retention (Pereira et al., 2009) and disfavor a prominent role played by α4β1–VCAM-1 in impeding migration across sinusoidal endothelium.

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Immature B cells egress BM by down-regulating CXCR4 by approximately twofold through a mechanism that was antagonized by antigen-induced BCR signaling, presumably after negative selection has occurred. In vitro studies using mouse primary immature B cells and a chicken B cell line reported that antigen-induced BCR signaling reduced B cell chemotaxis toward CXCL12, while having no detectable effect on CXCR4 surface expression (Guinamard et al., 1999; Brauweiler et al., 2007). Here, using two independent BCR transgenic mouse strains and WT (polyclonal) mice, we reliably measured BCR-induced CXCR4 expression in immature B cells in vitro and in vivo and a corresponding increase in B lineage interstitial motility and retention within BM parenchyma. The discrepancy between these findings could be related to an inadequacy of the in vitro systems for measuring antigen-coated immature B cell chemotaxis. However, our experiments are consistent with the observation that CXCR4 expression was increased in immature B cells in multiple murine models of lupus (Wang et al., 2009a), given that these cells encounter self-antigens during development in BM (Li et al., 2002). It will be valuable to analyze the cellular composition and biological significance of the BM niches that attract self-reactive immature B cells.

Even though the S1P pathway contributes to promote hematopoietic cell egress from BM, namely NK cells, monocyte/osteoclast precursors, eosinophils, and immature B cells (Walzer et al., 2007; Jenne et al., 2009; Allende et al., 2010; Pereira et al., 2010; Sugita et al., 2010), its relatively minor contribution is in agreement with a marginally detectable S1P gradient between BM parenchyma and sinusoids (Jenne et al., 2009). The BM sinusoids are fenestrated and allow passive flow of circulatory fluids into the parenchyma (Tavassoli and Yoffey, 1983; Mazo et al., 1998). Interestingly, the few observed examples of B lineage cell egress from BM parenchyma into sinusoids occurred exclusively in areas where vascular leakage was readily observed. This observation raises the possibility that S1P receptors promote BM egress from areas in parenchyma that are less perfused. Whether the fenestrated nature of the BM sinusoidal endothelium is a constitutive feature or a regulated process remains unknown.

The mechanical forces responsible for purging sessile B cells from BM are not entirely clear, but such mechanisms likely promote the egress of other hematopoietic cell subsets, including poorly motile red blood cells. The dependency on integrin-mediated adhesion for motility in BM demonstrates that developing B cells adopt similar mechanisms to those used by leukocytes during migration on luminal endothelial surfaces under shear stress generated by blood flow (Alon and Feigelson, 2009). The sudden morphological changes observed immediately after CXCR4 and/or α4 integrin blockade are also reminiscent of the cell shape changes seen in T cells exposed to lymph flow in lymphatic sinuses (Grigorova et al., 2009). Immature B cells likely adapted to haptokinetic migration for retention and controlled migration in shear-rich three-dimensional environments such as BM, and possibly the spleen. Consistent with this model, marginal zone B cells move between blood-bathed splenic red pulp and bloodless follicles in the white pulp and also rely on integrin-mediated adhesion for their retention in this compartment (Arnon et al., 2013). The observation that B lineage cell exit occurred from areas of intense parenchymal perfusion, and that several B cells were largely nonmotile and remained rounded during reverse transmigration, suggests that drainage of interstitial fluids back into circulation via collecting sinusoids facilitates BM export of B lineage cells and possibly other hematopoietic cell types, including red blood cells. In agreement with this model, neutrophils are also poorly motile in BM and are stationed near or at BM exit sites for several minutes before exiting into the sinusoidal lumen (Devi et al., 2013).

Whereas CXCR4-deficient pro-B and pre-B cells were easily detected in blood and spleen, as expected (Ma et al., 1999; Ueda et al., 2004), their numbers in BM were only slightly reduced when compared with immature B cell subsets. These data indicate that early B cell subsets are less efficiently mobilized from BM than immature B cells and suggest that they use other retention mechanisms. However, the majority of GFP^+ B lineage cells (which include pro-B and pre-B cells) remained predominantly sessile, exhibited a rounded morphology, and seemed largely nonadherent to the extracellular matrix when they lacked α4β1-mediated adhesion to VCAM-1. An alternative and attractive possibility is that pro-B and pre-B cells cannot undergo efficient reverse transmigration. Electron microscopy studies of femoral marrow of rats, rabbits, and guinea pigs documented extensive evidence of intimate interactions between immature lymphocyte pseudopodia and cell processes between adjacent sinusoidal endothelial cells (Tavassoli and Yoffey, 1983). In some instances, though not infrequent, lymphocyte pseudopodia were seen extending through endothelial cells (Hudson and Yoffey, 1966). We also observed few examples of WT and PTX-expressing B cells elongating during reverse transmigration through sinusoids. These observations suggest that reverse transmigration across some areas of the sinusoidal endothelial wall requires an active process. Our study reveals that it is independent of GPCR signaling.

Thymocyte egress is directly linked to TCR signaling intensity through AKT/FOXO1 inhibition of KLF2-dependent $S1PR_1$ expression (Hart et al., 2012), which likely prevents premature egress of autoreactive T lymphocytes (Zachariah and Cyster, 2010). Similarly, immature B lymphocyte egress is sensitive to BCR signaling intensity as it controlled CXCR4 expression. However, even when CXCR4 was overexpressed in developing B cells, a few immature B cells expressing high amounts of CXCR4 were detected in blood and spleen. Likewise, small numbers of B cells and neutrophils can be found in PB of WHIM patients, in which CXCR4 is constitutively active because of mutations that prevent its desensitization. Finally, autoreactive immature B cells can still egress from mouse and human BM, albeit inefficiently (Hartley et al., 1993; Wardemann et al., 2003). Combined, these data suggest that the BM architecture and its fenestrated vasculature generate a cell egress–permissive environment that contrasts with rather impermeable egress sites from the thymus. We suggest

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that the passive mode of leukocyte egress from BM evolved to facilitate the release of highly heterogeneous leukocyte subsets that include nonmotile cells, at the expense of some premature exit of developing B lymphocytes. This fundamental difference between T and B lymphocyte egress strategies from primary lymphoid organs possibly imposed an evolutionary pressure for additional extramedullary B cell developmental checkpoints (Carsetti et al., 2004).

MATERIALS AND METHODS

Mice. Adult C57BL/6 (CD45.2), Boy/J (CD45.1), *Itgb1*$^{Fl/Fl}$, *Lyz2*$^{Cre/+}$, *Cd19*$^{Cre/+}$, and *Rosa26*$^{FloxSTOP\text{-}ZsGreen/+}$ (Madisen et al., 2010) mice were from obtained from The Jackson Laboratory. MD4 and MD4.Bcl2 transgenic mice were from an internal colony (J.G. Cyster). B1-8 transgenic mice were provided by A. Haberman (Yale University, New Haven, CT). *Cxcr4*$^{Fl/-}$ and *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ (Regard et al., 2007) mice were crossed with *Mb1*$^{Cre/+}$ mice (Pelanda et al., 2002) to generate *Cxcr4*$^{Fl/-}$ *Mb1*$^{Cre/+}$ and *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Mb1*$^{cre/+}$ mice (all provided by J.G. Cyster). *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ mice were crossed to *Lyz2*$^{Cre/+}$ and *Il7r*$^{Cre/+}$ to generate *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Il7r*$^{Cre/+}$, *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Lyz2*$^{Cre/+}$, and control littermates. *Il7r*$^{Cre/+}$ mice were provided by H.-R. Rodewald (German Cancer Research Center, Heidelberg, Germany). For intravital imaging, *Cxcr4*$^{Fl/-}$, *Itgb1*$^{Fl/Fl}$, and *Rosa26*$^{FloxSTOP\text{-}PTX/+}$ *Mb1*$^{Cre/+}$ were crossed with *Rag1*$^{GFP/+}$ mice (Kuwata et al., 1999). Mice were maintained under specific pathogen–free conditions and used according to the protocols approved by the Yale University Institutional Animal Care and Use Committee.

Two-photon intravital imaging. Mice were anesthetized with ketamine/xylazine and immobilized on a custom-built stage. Laser-scanning microscopy images were collected using a BX61WI fluorescence microscope (Olympus) and a 20× 0.95NA water immersion objective (Olympus) and dedicated single-beam TriM Scope II (LaVision Biotec) controlled by IMSpector software. The microscope was outfitted with a Chameleon Vision II Ti:Sapphire laser (Coherent) with pulse precompensation. For 4D analysis of cell migration, stacks of 13–17 optical sections with 3-µm z spacing were acquired every 20 or 30 s for 30 min with the laser tuned to a wavelength of 845–875 nm. Pharmacological antagonists AMD3100 (Tocris Bioscience), 4F-benzoyl-TN14003 (a gift from H. Tamamura, Tokyo Medical and Dental University, Tokyo, Japan), and anti–VCAM-1 (clone M/K2; Bioexpress Inc.), anti-α4 (clone PS/2; LygoCyte Pharma Inc.), and anti-CXCL12 (clone 79014; R&D Systems) blocking antibodies were injected i.v. 1–5 min before imaging. Videos in which significant tissue drifting was detected were excluded from analyses. A few videos showed small tissue drifting, which was computationally corrected with Imaris software tool "Correct Drift" (Bitplane).

The axis ratio of parenchymal GFP$^+$ cells was calculated by measuring the distance between the two furthest GFP$^+$ points on the x and y planes of individual cells after collapse of the z plane using Imaris. Parenchymal GFP$^+$ cells that were distinguishable from neighboring cells were selected for analysis to avoid miscalculation of the axis ratio of a single GFP$^+$ cell.

Tissue preparation, cell enumeration, and antibodies. BM cells were flushed from femurs and tibias in DMEM (Cellgro) containing 2% FBS (Invitrogen), 5% of antibiotics (Cellgro), and Hepes (Cellgro). PB was collected from the portal vein, and erythrocytes were lysed with NH_4Cl, $KHCO_3$, and EDTA. Spleen was dissociated in 5 ml DMEM containing 2% FBS, 5% of antibiotics, and Hepes using a cell strainer (Thermo Fisher Scientific). Cells were counted with a Coulter Counter (Beckman Coulter). B cells were identified by staining with anti-B220 (RA3-6B2) or CD19 (1D3), anti-IgM (11/41), anti-IgD (11-26c.2a), and anti-CD93 (AA4.1) antibodies. Monocytes and granulocytes were stained with Ly6C (HK1.4), CD11b (M1/70.15), and CD115 (AFS98) antibodies, and NK cells were stained with CD3e (145-2C11) and NK1.1 (PK136) antibodies. Dead cells were excluded by staining with DAPI. Cells were analyzed by FACS (LSRII; BD).

Measurement of FITC-conjugated dextran dye perfusion into the parenchyma. Dye perfusion was measured by selecting two regions of interest in the BM parenchyma and FITC-conjugated labeled sinusoids using Imaris. A surface was applied to the green channel to generate voxels in regions of interest within the BM parenchyma and sinusoids over the course of the video. Perfusion of FITC-dextran into the parenchyma was assessed by measuring the number of voxels that appeared in the regions of interest over the course of a 29-min video.

Calculation of interstitial flow rate of dye perfusion into the BM parenchyma. Analyses of interstitial fluid flow were performed as described previously (Iliff et al., 2012; Egawa et al., 2013). In brief, flow rate within BM parenchyma was measured by injecting FITC-dextran (500 kD) or BSA–Texas red i.v. into anesthetized mice. Mice were then immediately imaged by two-photon IVM. The volumetric flow rate in the BM parenchyma was quantified by first applying a surface to the appropriate channel. Next, a three-dimensional box of a fixed volume was applied to three regions of interest. The instantaneous flow rates (on average six time points) of dye before the three-dimensional box was filled were used to calculate the mean instantaneous flow rate. The instantaneous flow rate ($\mu m^3/s$) was calculated by taking the difference between volumes from consecutive time points and dividing this difference by time (60 s).

In vivo labeling of BM sinusoidal B cell subsets. BM sinusoidal cells were labeled by injecting i.v. 0.3 µg phycoerythrin-conjugated rat anti–mouse CD19 (1D3) in 200 µl PBS. 2 min after, mice were sacrificed in a CO_2 chamber.

BrdU and PTX treatment. Mice were treated with 1 mg/ml BrdU (BD) i.v. and administered 1 mg/ml BrdU (Sigma-Aldrich) in drinking water 24 h before analysis. 1 µg PTX (List Bio Labs) was injected i.v. 24 h before analysis. BrdU-labeled cells were identified staining with anti-BrdU FITC according to the manufacturer's protocol. B cells were identified by staining with anti-B220 (RA3-6B2) or CD19 (ebio1D3), anti-IgM (11/41), anti-IgD (11-26.c.2a), and anti-CD93 (AA4.1). Monocytes, NK cells, and granulocytes were identified by staining for anti-Ly6c (HK1.4), anti-CD11b (M1/70), anti-CD3ε (145-2C11), anti-NK1.1 (PK136), and anti-CD115 (AFS98). After staining, cells were analyzed by FACS (LSRII).

BM chimeras. Approximately 1.5×10^6 total BM cells from Ly5.1$^+$ donors were mixed with 1.5×10^6 total BM cells from adult Boy/J (Ly5.2$^+$) mice and were transferred into adult Boy/J mice that had been exposed to two rounds of 6.35 Gy separated by 3 h. Chimeras were analyzed at least 6 wk after reconstitution.

Transwell migration assays. Chemotaxis assays were performed using 10^6 BM or spleen cells incubated for 30 min with 1× DMEM containing 0.5% fatty acid–free BSA (EMD Biosciences), 5% of antibiotics, L-glutamine (Cellgro), and Hepes. Cells were then allowed to migrate through 5-µm-pore–sized transwells (Corning) toward soluble CXCL12 (R&D Systems), 2-AG (Cayman), or CXCL13 (PeproTech) for 3 h at 37°C. Cells were collected, stained, and resuspended in 40 ml of staining buffer and analyzed by flow cytometry for 40 s.

Retroviral BM transduction. The R334X mutation in CXCR4 cytoplasmic tail was cloned into mammalian retroviral vector (pMSCV) upstream of an IRES-truncated GFP cassette as a reporter. Sanger DNA sequencing reaction verified truncated CXCR4 sequences. Phoenix 293T cells were transfected with MSCV retroviral constructs with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For mutant *Cxcr4* (R33x) transduction of hematopoietic stem cells, BM cells were harvested 5 d after i.p. injection of 5-fluoracil (Sigma-Aldrich) and cultured with IL-3, IL-6, and 100 ng/ml mSCF (PeproTech). BM cells were then spin-infected twice with a retroviral constructs and injected i.v. into lethally irradiated Boy/J recipients. Mice were analyzed at least 5 wk after reconstitution.

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In vitro antagonist treatments. 10^6 BM or spleen cells were treated with saline (0.9% NaCl) or AMD3100 for 1 h at 37°C. Cells were then removed from incubation and used in transwell migration assays.

BCR stimulations in vivo and in vitro. BM cells isolated from femurs and tibias were prepared at 10^7 cells/ml in DMEM containing 10% FBS, 10 mM Hepes, and a cocktail of penicillin and streptomycin (50 U/liter and 50 µg/liter, respectively). Approximately 2×10^6 cells were left unstimulated or stimulated with 20 µg/ml F(ab′)2 fragment goat anti–mouse IgM (Jackson ImmunoResearch Laboratories, Inc.), with 20 µg/ml HEL (Sigma-Aldrich), or with 1 mg/ml NP-BSA (Biosearch Technologies, Inc.), at 37°C and 5% CO_2 for the indicated time points in the Fig. 6 legend.

MD4 and MD4.Bcl2 transgenic mice were stimulated with 10 mg HEL for 6 h. Mice were injected with 0.3 µg phycoerythrin-conjugated rat anti–mouse CD19 (1D3) in 200 µl PBS before sacrifice. Cells isolated from femur and tibia BM, PB (portal vein), and spleen were counted in a Coulter Counter and stained to detect B cell subsets, as previously described in Tissue preparation, cell enumeration, and antibodies.

Statistics. Student's two-tailed *t* test or χ^2 test was performed using GraphPad Software Prism 6 or Microsoft Excel. A value <0.05 was considered significant.

Online supplemental material. Video 1 shows intravital imaging of developing B cell migration before and after treatment with AMD3100 (25 min). Video 2 shows intravital imaging of *Cxcr4*-deficient developing B cell migration (30 min). Video 3 shows intravital imaging of *Cxcr4*-deficient developing B cell migration before and after treatment with AMD3100 (30 min). Video 4 shows intravital imaging of developing B cell migration before and after treatment with anti–VCAM-1 (30 min). Video 5 shows intravital imaging of *Itgb1*-deficient developing B cell migration (30 min). Video 6 shows intravital imaging of developing B cell migration before and after treatment with anti–VCAM-1 (30 min). Video 7 shows intravital imaging of FITC-conjugated dextran perfusion into the BM parenchyma (29 min). Video 8 shows intravital imaging of PTX-expressing B lineage cell migration in BM (30 min). Video 9 shows intravital imaging of motile PTX-expressing B lineage cell migration in BM (30 min). Video 10 shows intravital imaging of WT and PTX-expressing B lineage cell egress from BM. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20140457/DC1.

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Immunity

Hematopoietic stem cell niches control multipotent progenitor differentiation

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Abstract:	Hematopoietic stem cells (HSC) self-renew in bone marrow niches formed by CXCL12+ mesenchymal progenitor and endothelial cells. Here, we show that hematopoietic multipotent progenitors (MPPs) encounter lineage-instructive differentiation signals in HSC niches. Conditional deletion of CXCR4 in MPPs profoundly reduced differentiation into common lymphoid progenitors (CLPs), which significantly decreased lymphopoiesis. CXCR4 was required for CLP positioning near IL-7+ cells and, consequently, for optimal IL-7R signaling. IL-7+ cells form a large subset of CXCL12-abundant reticular mesenchymal progenitor cells capable of differentiation into osteoblasts and adipocytes, and a minor subset of BM sinusoidal endothelial cells. Conditional Il7 deletion in mesenchymal progenitor cells dramatically reduced B-lineage committed CLPs, whereas Il7 deletion from endothelial cells specifically reduced preB cells. Our studies demonstrate that CLPs rely on positional cues to encounter short-range lymphopoietic signals provided by CXCL12+ mesenchymal progenitors. Thus, HSC maintenance and multilineage differentiation are distinct cell lineage decisions controlled by HSC niches.

From: Iannis Adamopoulos [iannis@hotmail.co.uk]
Sent: Friday, September 18, 2015 12:56 PM
To: Pereira, Joao
Subject: Osteoimmunology -Invited Review

Dear Dr. Pereira:

I am happy to inform you that your manuscript, "Inflammatory cell migration in rheumatoid arthritis" has been provisionally accepted for publication in Clinical Reviews in Allergy and Immunology.
Your article is now under editorial processing and will appear most likely the winter issue of December 2015.
Thank you for your interest in CRAI. I hope that we have the opportunity to work together again in the future.
Do not hesitate to contact me directly should you have any questions.
Iannis Adamopoulos Guest Editor Clinical Reviews in Allergy and Immunology

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Inflammatory cell migration in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that primarily affects the joints. Self-reactive B and T lymphocytes cooperate to promote antibody responses against self proteins and are major drivers of disease. T lymphocytes also promote RA independently of B lymphocytes mainly through the production of key inflammatory cytokines, such as IL-17, that promote pathology. While the innate signals that initiate self-reactive adaptive immune responses are poorly understood, the disease is predominantly caused by inflammatory cellular infiltration and accumulation in articular tissues and by bone erosions driven by bone-resorbing osteoclasts. Osteoclasts are giant multinucleated cells formed by the fusion of multiple myeloid cells that require short-range signals for undergoing differentiation. The recruitment and positioning of osteoclast precursors to sites of osteoclast differentiation is an important point of control for osteoclastogenesis and bone resorption and are reviewed. Furthermore, the pathways of osteoclast differentiation under homeostasis and driven by inflammatory signals are also reviewed.

Index entries/Keywords: Rheumatoid arthritis, osteoclasts, osteoclast precursors, cell migration

Introduction

Risk for inflammatory autoimmune diseases, like rheumatoid arthritis (RA), is influenced by genetic variation and environment. Genetic risk loci for autoimmune diseases have been identified by GWAS [1] and recently genetic fine mapping studies coupled with epigenomic analyzes have begun to identify putative causal variants [2,3]. Multiple disease-associated single nucleotide polymorphisms (SNPs) were mapped to genes and regulatory regions that are active in a cell-specific manner. These analyses revealed RA-associated SNP enrichment in genes and regulatory regions active in B cells, T cells, and to a lesser extent, monocytic cells [2,3]. This work provides a contextual framework for understanding the genesis of human autoimmune disease that can be applied to present and future studies in animal models. It also places the adaptive immune system front and center in the pathogenesis of RA.

Pathogenesis of RA

Rheumatoid arthritis is a chronic inflammatory disease characterized by an accumulation of inflammatory leukocytes in the synovium and articular tissues, and autoantibodies (rheumatoid factor and anti-citrullinated protein antibodies (ACPA)). The disease is also comprised of bone and cartilage tissue destruction and by other systemic features presumably caused by flares of inflammation. Even though RA is a common autoimmune disease that has been studied for several decades now, it is still unclear why articular tissues are predominantly attacked. The cellular components of the articular space include

cells of the innate and adaptive immune system, chondrocytes, fibroblast-like synoviocytes, and bone remodeling cells, i.e. osteoclasts and osteoblasts. The accumulation of inflammatory leukocytes in articular tissues is a hallmark feature of RA, but this accumulation is thought to occur primarily because of cellular recruitment into inflamed tissues rather than as a consequence of local leukocyte proliferation [4].

The adaptive immune response in RA has been a focus of intense research over the last several decades. The strongest genetic link to RA involves SNPs in the Human Leukocyte Antigen (HLA)-DRB1 gene that likely alter T cell repertoire selection and antigen presentation, amongst other possibilities. The B cell repertoire is also constrained in RA patients, in which defects in early B cell tolerance checkpoints have been described [5], but for which there is still little mechanistic insight. Numerous antigens have been described in RA patients and in RA mouse models, and they include antibodies against the immunoglobulin Fc region (rheumatoid factor) and against citrullinated proteins (ACPA). Citrullination is a post-translational modification in which arginines are deiminated by peptidylarginine deiminases. ACPA are not only prevalent biomarkers for RA diagnosis, they also predict the development of the more debilitating erosive aspects of the disease. To date, it still remains unclear if specific autoantigen epitopes are key for the pathogenesis of RA, but autoantigens are clearly essential for the adaptive immune response in the pathogenesis of RA.

Numerous citrullinated polypeptides have been detected in the synovium of RA patients, populating a “citrullinome” that is comprised of both intracellular

and extracellular antigens ranging in size from ~14-100 KDa [6]. ACPAs can be detected years before disease onset and are likely important for shaping the development of disease [7-9]. In support of this possibility it was recently shown that immune complexes with citrullinated histones are potent activators of myeloid cells (macrophages and neutrophils) and induce TNFα secretion [10].

Evidence from several mouse models of inflammation in the joint and of inflammatory arthritis provides valuable insight into the spatio-temporal dynamics of the antibody response [11]. The K/BxN transgenic mouse is a model that allows studying the mechanisms whereby autoantibodies drive inflammation in the joint. In K/BxN transgenic mice there is spontaneous inflammation in the articular space that presents synovial pannus formation and bone and cartilage erosions [12]. K/BxN mice spontaneously produce autoantibodies against glucose 6-phosphate isomerase (GPI) that selectively deposit in the joints as immune complexes and trigger complement fixation [13]. Transfer of serum containing arthritogenic anti-GPI antibodies from K/BxN transgenic mice into naïve wild-type mice leads to the development of inflamed joints clinically and pathologically similar to what is observed in K/BxN transgenic mice [14]. Given that the generation of autoantibodies in the K/BxN model is crucial for disease development, effort has been made to identify the anatomical location(s) and kinetics of anti-GPI antibody production. The anti-GPI antibody response initially localizes to draining LNs and persists until later stages of the disease [15]. This indicates that draining LNs are involved in the initiation and perpetuation of the inflammatory arthritis antibody response in this model. Evidence in the clinical

setting for LN involvement in RA patients has long been appreciated, including data showing that immune responses develop in draining LNs prior to the appearance of arthritis symptoms [16]. Importantly, B cell depletion from the draining LNs (popliteal) ameliorates inflammatory arthritis [17,18], and B cell depletion therapies significantly reduce RA symptoms in humans [19].

The activation of the B cell antibody response in the LN requires two antigen-driven events that are segregated in distinct temporal and anatomical locations: 1.) antigen recognition by B cells in follicles 2.) antigen presentation by T-zone dendritic cells and subsequent T cell activation in the T cell zone. Relatively small soluble antigens <70 kDa can gain access to B and T cell areas of the LN by free diffusion via the follicular conduit network [20-22]. Larger antigens are transported into follicles through their initial capture by $CD169^{+}$ subcapsular sinus macrophages from the incoming lymph, and relay to B cells in follicles in a complement receptor-dependent manner. These large immune complexes become subsequently deposited on follicular dendritic cells (FDCs) where they persist for long periods of time [23-26]. The molecular sizes of autoantigens uncovered in arthritis patients suggests both mechanisms of antigen delivery to T and B cell niches in secondary lymphoid organs are likely to operate in RA. But, T cells can also be activated by DCs that may sample antigens in inflamed articular spaces and migrate to the draining LN for T cell priming. In RA patients, both mature and immature DCs have long been identified in synovium and synovial tissue by histology [27-32]. Plasmacytoid DCs have also been identified in rheumatoid synovium and synovial fluid [33-35].

Whether various DC subsets are recruited to the joints during the earliest stages of synovial inflammation remains unknown.

Shortly after antigen-engagement, activated B cells increase the expression of the T-zone homing chemokine receptor CCR7, and migrate to the T-B border where they interact with a subset of activated (cognate) T cells [36]. At this stage, activated B cells receive further differentiation signals from cognate T cells mostly through CD40/CD40L interactions. Importantly, SNPs in CD40 have been linked to RA [4]. Within 1-2 days after antigen engagement and CD40 signaling B cells increase EBI2 (GPR183) expression [37-40] and together with activated T cells, migrate to outer and inter-follicular areas in an EBI2-dependent manner. The oxysterol ligands of EBI2 (mostly 7α, 25-hydroxycholesterol, (7α, 25-OHC)) are presumably abundant in the follicle perimeter, including interfollicular regions [37-40]. Dendritic cells also express EBI2 and migrate to inter-follicular niches, and signals or cellular interactions occurring at these sites contribute significantly to the development of primary antibody responses by promoting plasmablast differentiation [41,42]. Later in the immune response a subset of activated T and B cells initiates germinal center responses where antibody affinity maturation, isotype switching, and long-lived plasma cell differentiation take place.

The GC reaction is thought to play an important role in RA. Follicular dendritic cells (FDCs) are critical for the formation of autoreactive GC reactions and development of joint inflammation in the K/BxN model [43]. In RA patients, T and B cells form aggregates in the articular space and can form ectopic GCs in

about 40% of patients [44]. Given that anti-B cell therapy (Rituximab) is inefficient at depleting antibody-secreting plasma cells, and variably affects antibody titers [19], its efficacy may be associated with other roles B cells play in the organization of ectopic lymphoid follicles and GCs, and in T cell activation through cytokine secretion and autoantigen presentation. These tertiary lymphoid structures do not form in most experimental mouse models of inflammatory arthritis, the exception being in the collagen-induced arthritis (CIA) model [45]. The fact that CIA displays many similar histophathological features with human RA has made it one of the most widely studied models of inflammatory arthritis [46]. In this model, type II collagen (an abundant protein in cartilage), is used as an antigen for immunizing mice, which leads to the development of inflammatory arthritis. CIA is often used to evaluate therapeutic interventions.

Inflammation in the synovial compartment

Despite many decades of intense research very little is still known about the kinetics of cell recruitment into synovial tissues in RA patients and in experimental inflammatory arthritis. In a permutation of the K/BxN inflammatory arthritis model, LaBranche et al. transferred autoreactive KRN TCR transgenic T cells into T cell-deficient mice (B6.TCR.cα$^{-/-}$H-2$^{b/g7}$) and were able to induce arthritic lesions that were synchronized and chronic in nature [47]. Interestingly, T cells were not detected in the joint but were present in the draining popliteal LN. Instead, monocyte/macrophage and neutrophil infiltration occurred early in

disease progression, and correlated with bone and articular erosions. The influx of monocytes and macrophages, whether by recruitment or local differentiation and activation, highly correlates with inflammation and tissue damage in RA patients [48-50]. Given that monocytes and macrophages secrete numerous chemokines and cytokines, they also could be involved in the recruitment of other inflammatory cells into the joint, such as neutrophils. Neutrophils are abundant in synovial tissues and fluid, and mouse models provide clear evidence for neutrophil involvement in disease. Neutrophil depleted mice are completely resistant to the K/BxN serum transfer model of inflammatory arthritis [51]. A study in an anti-type II collagen antibody induced inflammatory arthritis model reported that the initial cellular infiltrate to the synovium is mainly composed of neutrophils and macrophages [52], and depletion of neutrophils attenuates disease progression [52,53]. However, the mechanisms controlled by neutrophils that exacerbate disease remain poorly understood. Furthermore, whether macrophages also contribute to the onset of disease remains unclear.

Recruitment of innate immune cells

In the synovium and synovial fluid of RA patients numerous homeostatic and inflammatory chemokines with the potential to influence the trafficking of leukocytes into the tissue are upregulated. $CCR7^+$ mature DC ($CD83^+$ and DC-$LAMP^+$) present within synovial infiltrates of RA patients were found in close association with CCR7-ligand (CCL19 and CCL21) expressing cells, suggesting these chemokine pairs may play a role in the recruitment of DCs to the synovium

[32]. In this study immature DC ($CD1a^+$) expressed CCR6 and were in close association with CCL20-producing synovial cells [32]. In humans it was also recently reported that pDCs expressed CXCR3 and CXCR4 and accumulated in the inflamed joints of RA patients, [34]. Furthermore, the levels of CXCR3 ligands CXCL10, CXCL11, and of the CXCR4 ligand CXCL12 in the synovial fluid of RA patients were significantly increased when compared to osteoarthritis (OA) patients [34], suggesting the involvement of CXCR3 and CXCR4 in pDC homing or retention in inflamed joints. Besides pDCs, other DC subsets express functional CXCR4 [54], and CXCL12 was found to be highly expressed in synovial tissues [55,56] and synovial fibroblasts from RA patients [57].

In the K/BxN serum transfer model, neutrophils are absolutely required for disease progression [51]. Neutrophils express CXCR2 and its ligand CXCL2 is abundant in arthritic tissues including CXCL2 [58]. Furthermore, CXCR2 was crucial for disease progression in this model [58]. Importantly, blockade of CXCR2 in other animal models of arthritis quantitatively reduces disease severity and the recruitment of neutrophils [59-61]. Given that CXCR2 promotes neutrophil egress from bone marrow cavities [62], it is unclear if CXCR2 deficiency reduced inflammatory arthritis by impairing neutrophil recruitment to the synovium or by reducing their egress from bone marrow, or both. Using intravital 2-photon imaging of LysM-GFP mice and the K/BxN serum transfer model of arthritis, Wang and colleagues were able to visualize neutrophil recruitment to the inflamed synovial space [63]. Intriguingly, they observed that

neutrophils were often associated with monocytes at sites of extravasation, and global depletion of monocytes arrested neutrophil extravasation [63].

Recruitment of lymphocytes

Th1, Th17, and Treg cell types are present in inflamed articular tissues in RA patients, with Th1 cells being the most prevalent T helper cell subset [64,65]. Studies from mouse models in combination with data obtained from RA patients have provided some insight into the trafficking mechanisms for T cells in arthritis. CXCR3 and CCR5 are abundantly expressed by T cells isolated from the synovial fluid and synovial tissues of RA patients, and both receptors are preferentially expressed on Th1 cells [66-68]. In adoptive transfer experiments in a mouse adjuvant arthritis model, T cells deficient in CXCR3 were poorly recruited to inflamed joints, and CXCR3 blockade using antibodies inhibited T cell recruitment and reduced disease severity [69]. Consistent with these findings, the CXCR3 ligands CXCL10 and CXCL9 are abundant in the synovial fluid of RA patients [66] and, interestingly, myeloid DCs isolated from the synovial fluid of RA patients express higher CXCL9 and CXCL10 amounts than myeloid DCs isolated from peripheral blood [70]. CCR5 has also been suggested to contribute to the recruitment of T cells to the synovium. Several studies have reported that a single nucleotide polymorphism (SNP) resulting in a non-functional CCR5 allele was negatively associated with RA [71-74], but this finding was not confirmed in other studies [75,76]. In animal models CCR5 deficiency had no effect on disease outcome [77].

In RA patients, CXCR4 is expressed on memory $CD4^+$ T cells ($CD45RO^+$) infiltrating the synovium [68]. In mice, T cell specific deletion of CXCR4 impaired their infiltration into the joints and decreased the severity of collagen-induced arthritis [78].

CX_3CR1 is expressed on T cell subsets, and in RA patients the CX_3CR1^+ fraction of T cells is increased when compared to healthy controls [79]. The ligand for CX_3CR1, fractalkine (CX_3CL1), is expressed by synoviocytes and endothelial cells and is elevated in RA patients compared to OA patients [80,79,81]. In the collagen induced mouse model of arthritis, CX_3CR1 is also expressed on T cells infiltrating the synovium and CX_3CL1 is also upregulated [82]. Given that the depletion of CX_3CL1 in synovial fluid in animal models of inflammatory arthritis reduced overall chemotactic potency [83], this chemokine receptor pair likely guides the recruitment of T cells to inflamed synovium.

Th17 cells play a key role in the pathogenesis of RA and these cells express CCR6 [84]. CCR6-expressing cells were identified in synovial tissue from RA patients several years ago [85], and the CCR6 ligand CCL20 is robustly secreted by synovial fibroblasts isolated from arthritic joints (but not secreted by granulocytes and monocytes) [84,85]. CCR6 blockade suppressed the onset and severity of disease and reduced the number of $CD4^+$ T cells that infiltrated the joint [84], although the numbers of Th17 cells were not specifically reported.

Administration of FTY720, a high affinity agonist for sphingosine 1-phosphate (S1P) receptors that induces receptor internalization and renders targeted cells unresponsive to chemotactic gradients of S1P, was protective in

mouse models of inflammatory arthritis by reducing lymphocytic infiltration into the joints and bone erosions [86-88]. Lymphocytes (including T cells) [86-88] and monocytes [87] were sequestrated in secondary lymphoid organs and thus, were unavailable to egress into the circulation and migrate to arthritic tissues. Although bone marrow populations were not examined in either study, it highlights the importance of this pathway in lymphocyte circulation and trafficking in inflammatory arthritis. S1P levels are elevated in the synovium and synovial fluid of RA patients (even higher than serum levels) and could contribute to cellular recruitment and retention [89,90].

An essential component of the pathogenesis of RA is B cells, highlighted by the efficacy of B cell depletion therapy [91,92]. In RA, B cell production of ACPA is strongly predictive of erosive bone disease [93]. B cells can be found in abundant numbers organized into follicular structures in the synovium, and in $ACPA^+$ RA patients about 25% of IgG^+ B cells isolated from articular space encode antigen receptors that are specific for citrullinated antigens [94]. Whether self-reactive B cells differentiate within ectopic follicles and germinal centers in the synovium, or are recruited from draining LNs is not entirely clear.

The chemokine expression pattern on B cells isolated from the synovium of RA patients includes CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5 [95]. The levels of CCL19, CXCL12, and CXCL13 are all increased in the serum of RA patients [96], CCL19 is increased in the synovium of RA patients [97], and CXCL13 is expressed in ectopic germinal centers in the synovium of RA patients [98]. This combination of homeostatic and inflammation-associated

chemoattractant receptors suggests an active involvement in B lymphocyte recruitment and cell organization in synovial spaces.

Inflammation in the subchondral bone marrow

The synovium, and more recently LNs, represent the main focus of the inflammatory response in RA, but nearby compartments also develop inflammation including the subchondral bone marrow. The subchondral bone marrow is located under the subchondral bone that separates the articular cartilage from the bone marrow cavity. The bone marrow cavity is an important site of hematopoiesis but the subchondral bone marrow is populated by adipocytes and lacking abundant hematopoietic activity under homeostatic conditions. However, evidence indicates that subchondral bone marrow participates in the inflammatory response in RA. Magnetic resonance imaging data (MRI) from RA patients identifies edema throughout the bone marrow, even at stages where there is a low level of synovial inflammation [99,100]. Indeed, like synovitis, bone marrow edema is also predictive of subsequent pathologic bone erosion [101,102]. Subchondral bone marrow inflammation, including ectopic germinal center-like structures, has been found in the CIA mouse model [45,103,104]. The subchondral bone marrow undergoes significant changes during the course of RA, including infiltrating leukocytes [105-108] and development of bone resorbing osteoclasts (OC) on the subchondral surface [109,110]. Collectively, these studies indicate that the bone marrow

compartment may be important for the pathogenesis of RA, but this compartment has not yet been fully integrated into disease models.

Bone erosion in RA

Periarticular bone erosions are highly prevalent in RA and lead to joint deformities and impaired function [111]. As the only cell capable of bone resorption in the body, osteoclasts have a critical role in inflammatory disorders associated with bone erosions and focal bone loss, including RA [112]. In mouse models of inflammatory arthritis numerous studies have demonstrated that osteoclasts are absolutely required for bone erosions to occur [113-118]. Osteoclasts form early in disease progression in animal models and initially appear at the junction of synovial membrane and periosteal lining and within subchondral bone [119]. Even though osteoclasts can be detected on both the synovial and subchondral bone marrow sides of the joints, osteoblasts (bone synthesizing cells) are only detected on the subchondral side [110,120-122]. Therefore the mechanisms of osteoclast differentiation from these two sides may be distinct.

Osteoclast differentiation under homeostasis.

Osteoclasts are hematopoietic cells that differentiate from monocyte progenitors through sequential developmental stages. Although the exact identity of osteoclast precursors still remains unclear, studies have found that cells with osteoclastogenic potential in the bone marrow do not express lymphoid

cell surface proteins or the aM integrin (CD11b/MAC1), and instead express the macrophage colony stimulating factor-1 receptor (Cfms, CSF1R, CD115) and the stem cell factor receptor (cKit). These cells overlap with the macrophage/dendritic cell precursor (MDP) subset [123] and can also differentiate into monocytes, macrophages, and dendritic cells [124-127]. More recently, a clonogenic, monocyte- and macrophage-restricted progenitor cell (named cMoP) derived from the MDP was identified [128], but whether the cMoP subset retains osteoclast differentiation potential has not been defined.

The dominant cytokine that governs osteoclast differentiation in vitro and in vivo is RANKL, which signals through its receptor RANK [129-131]. Osteoblasts are key sources of MCSF (a cytokine required for cell survival and osteoclastogenesis) and RANKL [132], and RANKL expressed by osteocytes was recently shown to be critical for osteoclast differentiation in vivo [133]. RANKL is a type II transmembrane receptor and as such requires cell-cell interactions for engaging its receptor RANK expressed on osteoclast precursors. This predicts that positional cues are involved in the recruitment of osteoclast precursors to interact with $RANKL^{+}$ cells. For many years it has been appreciated that resorbing bone is chemotactic for osteoclast precursors [134]. Recently, osteoblasts were reported to express oxysterol-synthesis enzymes, namely cholesterol 25 hydroxylase (gene name *Ch25h*) and 25-hydroxycholesterol 7-alpha-hydroxylase (gene name *Cyp7b1*) [135]. They also secrete the oxysterol 7a, 25 dihydroxycholesterol (7a, 25-OHC), which acts as a potent chemotractant for the pertussis toxin sensitive Gai protein coupled receptor EBI2 [135].

Furthermore, EBI2 is abundantly expressed in murine osteoclast precursors, and its expression increases during osteoclast differentiation [135]. It was reported that EBI2 directs the migration of osteoclast precursors to bone endosteal niches and promotes osteoclastogenesis in vivo [135]. Besides its role in cell positioning at the endosteum, EBI2 signaling also contributes to promote osteoclast precursor motility within bone marrow parenchyma [135], possibly in synergy with CX3CR1 [136]. Importantly, deficiencies in EBI2 or CH25H resulted in similar decreases in osteoclast numbers and increased bone mass in vivo [135], indicating that 7a,25OHC is the main EBI2 ligand controlling osteoclastogenesis and bone mass homeostasis. In summary, this study provided direct in vivo evidence for EBI2 and oxysterols in osteoclast precursor recruitment to endosteal surfaces, and consequently in osteoclast differentiation [135].

The chemokine monocyte chemoattractant protein-1 (MCP-1) is also expressed by osteoblasts in vitro when cultured in inflammatory conditions [137-139] and the MCP-1 receptor, CCR2, is expressed on mouse and human osteoclast precursors [140,141]. These findings led to a model where bone proximal MCP-1, produced by osteoblasts, attracts osteoclast precursors to sites of osteoclast differentiation [142]. In support of this model, CCR2 deficient mice display a mild increase in bone mass and stability, likely due to a reduction in osteoclast differentiation [143]. In humans, a polymorphism in CCR2 (V64I) was associated with increased bone mineral density in middle aged men and postmenopausal women [144], but whether this polymorphism alters CCR2 signaling is unclear [145]. Altogether, the bone mass phenotype reported in

CCR2 deficient mice is mild and contrasts with the essential role of CCR2 in promoting monocyte egress from bone marrow, both in homeostasis and during systemic acute inflammation [146-148].

CCR1 and CCR5 have also been reported as abundantly expressed on human, mouse and rat osteoclast precursors, upregulated on osteoclasts, and promoting their chemotaxis in response to several of their ligands, as reviewed elsewhere [149]. Furthermore, CCR1 and its ligands significantly promote osteoclast differentiation in vitro and in vivo (particularly CCL9) and CCR1 deficient mice are mildly osteopenic [150,149]. Several CCR1 ligands are shared by CCR5, but no bone mass phenotypes in CCR5 deficient mice have been reported.

The chemokine receptor pair CX_3CR1 and its ligand CX_3CL1 are also functionally expressed during osteoclast differentiation, as reviewed elsewhere [149]. Interestingly, CX_3CL1 is produced by mouse osteoblasts [151] and CX_3CR1 deficient mice have a reduction in osteoclasts that results in a mild increase in bone mass [152]. Furthermore, functional inhibition of CX_3CR1 signaling provided protection from bone loss induced by irradiation [153], perhaps by disrupting CX_3CR1-mediated migration and retention in the bone marrow [136]. In summary, while these studies suggest that synergy between multiple chemokine receptors controls osteoclastogenesis, only EBI2 was shown to be directly required for osteoclast precursor positioning in bone endosteal niches (Figure 1).

In contrast to chemoattractants derived from bone-proximal niches, chemoattractants emanating from bone-distal niches can also influence the positioning of osteoclast precursors in the bone marrow. Furthermore, the balance between bone marrow retention and egress cues is likely an important point of control for osteoclast precursor positioning and differentiation. In favor of such a model is the fact that osteoclasts are predominantly in close proximity to blood vessels in bone marrow thus making it likely that osteoclast precursors encounter bone and blood vessel chemoattractants (Figure 2) [149].

Emerging evidence has provided insight into leukocyte migration and dynamic behavior in steady-state conditions, including the mechanisms controlling their egress from bone marrow. Leukocytes are thought to egress from bone marrow through a vast network of blood vessels often referred to as bone marrow sinusoids. The majority of bone marrow sinusoids are characterized by a single layer of endothelial cells often separated by small gaps and without a basement membrane. Several hematopoietic cells exit bone marrow parenchyma by moving towards bone marrow sinusoids in a chemoattractant-dependent manner. For example, natural killer cells, eosinophils and B lymphocytes express S1P receptors and migrate towards high concentrations of S1P within sinusoids [154-157]. Other hematopoietic cells, such as monocytes and neutrophils depend on chemokine receptors, namely CCR2 and CXCR2, for exiting bone marrow parenchyma, and CCR2 ligands are abundant around sinusoids [147,62,146]. Osteoclast precursors have also been shown to use S1P receptors for exiting bone marrow, and defects in S1PR

signaling alter osteoclast precursor differentiation [158,159]. CXCR4 is also expressed in osteoclast precursors and directs their migration towards CXCL12 in vitro [160,161]. CXCR4, and its ligand CXCL12, have a well-described role in the retention of multiple hematopoietic cell subsets in bone marrow, including hematopoietic stem and progenitor cells [162]. Even though CXCR4 is one of the most studied chemokine receptors, its role in steady-state osteoclastogenesis in vivo remains controversial. Several studies reported that osteoblasts express CXCL12 in vivo [163,164] and it was proposed that CXCR4 promotes osteoclast precursor migration towards sites of osteoclast differentiation in vivo [158,165]. However, studies using CXCR4 deficient and CXCL12 reporter mouse strains provided results that are not compatible with this model. For example, CXCL12 expression is highest in rare mesenchymal stromal cells capable of multilineage differentiation (osteoblasts, chondrocyte, and adipocytes) distributed in parenchyma and some adjacent to blood vessels [166-168]. Furthermore, CXCL12 expression is reduced by nearly 100-fold in osteoblasts in vivo [169], making it unlikely that CXCR4 would direct osteoclast precursors towards osteoblasts. In agreement with this possibility, osteoclast differentiation and bone resorption were increased when hematopoietic cells lack CXCR4 expression, [170], which favors a model where the balance between responsiveness to bone chemoattractants and to other chemoattractants influences osteoclast development in vivo (Figure 1).

Cannabinoid receptor-2 (CB2) is abundantly expressed in various hematopoietic cells, including monocytes/osteoclast precursors and it promotes

cell migration to its ligand 2-arachidonoylglycerol (2-AG) [171]. In vivo, 2-AG activity is likely high in (or near) bone marrow sinusoids given that CB2 promotes immature B lymphocyte positioning in these locations, a process that is also dependent on $\alpha 4\beta 1$ adhesion to VCAM-1 [172]. It is, thus, conceivable that monocytes and osteoclast precursors are similarly attracted and/or retained within bone marrow sinusoids via CB2 chemotactic activity. In support of this hypothesis, CB2 deficient mice exhibit low trabecular bone mass, and CB2 antagonists inhibit osteoclast differentiation in vitro [173,174]. Furthermore, in humans a single nucleotide polymorphism in *CNR2* (encoding CB2) was significantly associated with osteoporosis [175-177].

Leukocytes can exit bone marrow through mechanisms that are independent of pertussis toxin-sensitive Gαi protein coupled receptors, and presumably independent of chemoattractant gradient sensing and cell intrinsic motility [178]. B-lineage lymphocytes enforced to express pertussis toxin or deficient in CXCR4 expression were found to be largely non-motile within bone marrow cavities of live mice, and were rapidly mobilized from bone marrow parenchyma into blood [178]. It was also noted that the bone marrow parenchyma is under shear stress induced by plasma perfusion and interstitial fluid flow [178]. It is plausible that the highly fenestrated nature of the sinusoidal network in combination with plasma and interstitial fluid flow back to collecting sinusoids allows non-motile cells (e.g. red blood cells) to exit the bone marrow in a passive manner, and that such unconventional exit routes are used by essentially all leukocytes, including osteoclast precursors.

Osteoclast differentiation within inflamed synovial space.

The identity of osteoclast precursors in arthritis may be distinct from steady-state osteoclast precursors, but presumably belongs to the myeloid cell compartment. Myeloid cells are accumulated in synovial tissue and synovial fluid in RA [48,49]. Some studies have interrogated the phenotype of osteoclast precursors in inflammatory arthritis models. The hTNFα transgenic strain develops synovial hyperplasia and lymphocytic infiltrate, pannus formation, articular cartilage destruction, and osteoclast driven bone erosion [179]. In this model, a cell population expressing the aM integrin CD11b but not Gr-1 displayed osteoclastogenic potential, and this population was increased in the bone marrow and blood of hTNFα transgenic mice [180]. Another study utilizing the SKG model of spontaneous inflammatory arthritis identified a population of cells with osteoclastogenic potential that had low to negative expression for CD11b and expressed high levels of Ly6C [125], and these cells may overlap with cMoPs (Nevius and Pereira unpublished observations). Dendritic cells have also been reported to contain osteoclast differentiation potential. Specifically, immature DCs were able to form osteoclasts in response to MCSF and RANKL, and unidentified soluble factors in human synovial fluid increased the DC differentiation into osteoclasts. These findings indicate that DCs may contribute to arthritis not only by acting as antigen-presenting cells and promoting T cell activation, but also by their potential to differentiate into bone-resorbing osteoclasts [181,182]. Collectively these studies suggest that multiple myeloid cell populations contain osteoclast differentiation potential (Figure 3).

RANKL is expressed on activated T cells, B cells, DCs, and synovial fibroblasts, besides bone-producing cells, RANKL expressed on T cells [133,183] and B cells [184] is dispensable for osteoclast differentiation and skeletal development and maintenance under homeostatic conditions. However, in mouse models of inflammatory arthritis, and in RA patients, the expression of RANKL on T cells and synovial fibroblasts is robust [113,185-187]. In murine inflammatory arthritis it has been established that synovial fibroblasts support the conversion of $FOXP3^+$ Tregs into pathogenic Th17 cells, which express higher RANKL amounts than other $CD4^+$ T cell subsets [188]. Furthermore, Th17-derived RANKL is able to support osteoclastogenesis in vitro [188,189]. That the cytokine IL-17A is an important driver of osteoclastogenesis in arthritis has been appreciated for some time now [190], but the mechanisms of its action are still being elucidated. Interestingly, IL-17A has been shown to induce RANK on human and murine osteoclast precursors and promotes osteoclastogenesis [191-193]. In addition, some evidence suggests IL-17A may increase RANKL expression on fibroblastic synoviocytes and/or mesenchymal-derived cells [189]. A recent study has dissected the relative contributions of RANKL derived from T cells and synovial fibroblasts and found that synovial fibroblasts provide the relevant sources of RANKL in models of inflammatory arthritis [194]. In humans, RANKL blockade with denosumab reduces bone erosion possibly through inhibition of osteoclast differentiation and function [195]. In mice, RANKL-expressing Th17 cells have been reported to interact directly with osteoclasts in vivo. By fluorescently labeling osteoclasts and Th17 cells it was observed that

Th17 cells preferentially located in contact with mature osteoclasts and this interaction was partially dependent on RANKL [196]. Finally, by using a pH sensitive fluorescent probe and an indicator of local pH decreases associated with osteoclast H^+ secretion and bone resorption it was observed that Th17 and osteoclast interactions occurred in sites of active bone resorption [196]. Collectively through these studies a picture emerges in which Th17 derived IL-17A regulates the expression of RANKL on synovial fibroblasts, enhances the differentiation of osteoclasts in the inflamed joint, and exacerbates bone resorption in vivo (Figure 3).

As mentioned previously, MCSF and RANKL are critical cytokines for osteoclastogenesis, and while MCSF is a soluble cytokine, RANKL is a type II membrane protein. Therefore osteoclast precursors in the joint presumably receive RANK signaling directly from fibroblastic synoviocytes, and possibly from T cells, osteoblasts, or from interaction with osteocyte processes at the bone surface (Figure 3). Given that osteoclasts can form bi-directionally in the joint (synovium and bone marrow), the specific cell type presenting RANKL (and other cytokines) on the synovial or bone marrow side may differ, and may be under the influence of distinct cell recruitment mechanisms.

RANKL independent osteoclast differentiation in RA

It is now appreciated that in inflammatory settings osteoclast differentiation can occur independently of RANKL signaling [197]. Inflammatory cytokines such as TNFα [198], TNFα with IL-6 [199], and IL-1α [200] can induce osteoclast

differentiation when in the presence of MCSF, but non-inflammatory cytokines like TGFβ have also been shown to trigger osteoclast differentiation [201]. Collectively these data identify cytokine-driven alternative pathways of osteoclast differentiation not dependent on the RANK/RANKL signaling pathway. Many of these pro-osteoclastogenic cytokines can be produced by macrophages, as reviewed elsewhere [197], and synovial macrophage numbers correlate with severity of inflammatory activity in RA [202]. Although pro-osteoclastogenic cytokines are not exclusively produced by synovial macrophages, these cytokines are present in the synovial milieu and may contribute to alternative pathways of osteoclast differentiation. Recent work from Harre and colleagues found that ACPAs enhanced the differentiation of osteoclasts in vitro [203]. They further showed that administration of ACPAs to lymphocyte deficient $Rag1^{-/-}$ mice was able to increase osteoclast numbers and reduce bone mass in vivo [203]. The mechanism by which ACPAs promote osteoclast differentiation is in part dependent on the Fc sialylation state of IgG in immune complexes [204]. Alternative pathways of osteoclast differentiation, driven by the unique inflammatory cytokine milieu (e.g. TNFα) and pathogenic autoantibody responses likely synergize with the dominant RANK/RANKL signaling pathway to induce bone erosions associated with RA. Indeed, TNFα induces RANKL expression on stromal cells via IL-1α signaling and enhance RANKL-dependent pathways of osteoclast differentiation [205].

Recruitment of osteoclast precursors in RA

It is clear in murine inflammatory arthritis and human RA that osteoclast precursors populate the synovium and synovial fluid. Conceptually, osteoclast differentiation in RA is likely controlled by multiple guidance cues balancing osteoclast precursor retention in bone marrow, migration from the bone marrow to egress sites, and migration into tissues, i.e. the synovium (Figure 3). The chemotactic signals guiding cells to these sites are incompletely defined and are of great interest. Some studies indicate that the cytokine TNFα, which is abundant in synovial tissue, can expand osteoclast precursors in inflammatory arthritis models and can also induce the mobilization of osteoclast precursors from the bone marrow into the periphery [206,207,180]. Given that in RA patients S1P levels are elevated in the synovium and synovial fluid (even higher than serum levels) [89,90], S1P receptor signalling could contribute to monocyte recruitment and retention in the joints. Consistent with this hypothesis, blockade of S1P signaling by administration of FTY720 reduces lymphocyte [86-88] and monocyte [87] migration to the joints.

Human and mouse monocytes/osteoclast precursors and osteoclasts also express functional CCR1, CCR5, and CCR2. The CCR2 and MCP-1 chemokine receptor pair not only promotes monocyte egress from bone marrow under homeostatic and inflammatory conditions [146-148], but MCP-1 expression is increased in inflamed synovial fluids [208,209]. Therefore, these collective reports suggest that CCR2 attracts monocytes/osteoclast precursors from blood circulation to inflamed joints. It remains to be completely dissected the relative

contribution of CCR2 in the egress of monocytic cells and in the recruitment of those cells into tissues like the inflamed synovium. Potential sources for MCP-1 include osteoblasts and mesenchymal stromal cells.

Early studies in mouse models showed that the severity of inflammatory arthritis is mitigated by administration of receptor antagonists to CCR2 and CCR5 [210,211]. It was subsequently elucidated that the kinetics of CCR2 blockade are crucial for a beneficial outcome [212]. However, genetic deletion of these receptors had an opposite effect. Mice genetically deficient in CCR2 and CCR5 displayed either enhanced severity of inflammatory arthritis (including enhanced monocyte recruitment) or had no effect on disease outcome, respectively [77]. More recently, inflammatory arthritis induced in CCR2 deficient mice were described to have increased Th17 cells, which may partially explain the disease exacerbation [213]. The blockade of another GPCR, CCR1, in inflammatory arthritis mouse models showed both inhibition of chemotaxis and reduction of inflammation [214,215,211,216].

The promising data from mouse models prompted the development and testing of CCR1, CCR2, and CCR5 antagonists as a therapeutic approach for the treatment of RA [217-221]. However, the results of the clinical trials were variable and did not show an overt efficacy in the treatment of RA patients. This could be in part due to the redundancy of the chemokine receptor and chemokine system. For example, CCR1 has 13 distinct chemokine ligands, some of which are shared ligands for CCR2, CCR3, CCR5, and CCR10. There are additional

chemokine receptors that have been suggested to regulate the recruitment of monocytes/osteoclast precursors in RA.

Other chemokine receptor pairs that may regulate the recruitment of osteoclast precursors to the bone surfaces in homeostasis may also play a role in inflammatory conditions, like RA. For instance, CXCL12 is highly expressed in synovial tissues [55,56] and synovial fibroblasts from RA patients [57] and the migration of monocytes to supernatants from synovial fibroblasts was significantly decreased after neutralization of CXCL12 [222]. In the context of RA, CB2 is more abundantly expressed in the synovial tissues of RA patients, and administration of a selective CB2 antagonist in a mouse model ameliorated inflammatory arthritis and bone destruction [223]. Although the distribution of 2-AG within bone marrow has not been exactly defined, the fact that CB2 signaling occurs in the vicinity of (or within) bone marrow sinusoids under normal homeostatic conditions, and that it can be detected in synovial tissues, suggests that 2-AG may act as a chemoattractant or a retention signal. Finally, the neutralization of the CX_3CR1 ligand, CX_3CL1 (fractalkine), in synovial fluid in murine inflammatory arthritis reduced overall chemotactic potency [83]. Blockade of CX_3CR1 in a murine model decreased the recruitment of monocytic cells to synovial tissues and decreased disease incidence and severity [82]. CX_3CL1 is elevated in RA patients compared to OA patients [81,80] and CX_3CL1 has been shown to be upregulated in mouse bone marrow endothelial cells in inflammatory conditions, such as full body irradiation [153].

CXCR2 and CXCL2 have also been implicated in inflammatory bone remodeling [224]. In osteoclast precursors, CXCL2 is induced by RANKL and promotes their chemotaxis to CXCL2 [224]. The expression of CXCL2 was increased in the synovial fluid of RA patients and CXCL2 significantly enhanced bone resorption in vivo [224]. Therapeutic neutralization of this chemokine receptor pair had positive results in animal models of arthritis [225,60,226], possibly owing to the inhibition of CXCL2-mediated recruitment of osteoclast precursors to inflamed joints. In the K/BxN serum transfer model, the relevant chemokine receptor for arthritis progression was shown to be CXCR2, specifically for the recruitment of neutrophils [58]. But this study does not preclude an important role for CXCR2 in the recruitment of osteoclast precursors [58].

Under homeostasis, EBI2 and CH25H are required for osteoclast precursor recruitment and osteoclast differentiation. But, EBI2 and CH25H are also required for the generation of antibody responses, and have also been implicated in a variety of inflammatory diseases. SNPs in Ebi2 or in regulatory elements of *Ebi2* expression are associated with cardiac inflammation, type I diabetes and inflammatory bowel disease [227-229]. Furthermore, *Ch25h* is one of the most induced interferon-stimulated genes [230]. Future studies should determine if CH25H activity is increased in the inflamed articular space, and if EBI2 ligands play roles in inflammatory cell recruitment and osteoclast differentiation in inflamed joints.

While many studies interrogated functional roles for chemoattractant receptors in osteoclast differentiation and bone erosions in RA patients, and mouse models of RA, little remains understood about the manner in which they contribute to disease. It is likely that osteoclast precursor differentiation within inflamed synovium requires sequential chemotactic signals: at the first stage cells must be captured from blood circulation and extravasate into the synovium. Subsequently, osteoclast precursors likely follow chemotactic gradients that not only promote retention within the articular space but may also direct their migration towards sites of osteoclast differentiation. It is possible that redundancy between chemokine and other chemoattractants control such multi-step process of osteoclast differentiation.

Inflammation induced changes in bone homeostasis

Hematopoiesis has been proposed to be regulated locally by cytokines [231]. Cytokines are capable of instructing hematopoietic lineage choice by acting on 1) HSC, 2) Hematopoietic progenitor cells (HPC), and 3) mesenchymal stromal cells that support HSC. Furthermore, inflammatory cytokines, such as type I interferons may also function as rheostats of HSC proliferation [232,233]. During stress response, HSC enter in cycle and self-renew but when homeostasis is reestablished, HSC return to a quiescent state [234].

The inflammatory cytokine signature of RA includes several cytokines that have profound effects in hematopoiesis by biasing cell lineage choices. Two of the most notorious are TNFα and IL-1, and these cytokines can induce

hematopoietic shifts that favor the production of inflammatory, innate-type cells, such as neutrophils and monocyte-lineage cells, at the expense of lymphopoiesis [235,236]. Of note, systemic TNFα increases the number of $CD11b^{high}$ osteoclast precursors in circulation [237], and this effect may in part be due to an increase in osteoclast precursor proliferation and differentiation in bone marrow [180]. Such inflammation-induced shifts in hematopoiesis are most likely dependent on local G-CSF production, although it is possible that other inflammatory signals contribute to this effect [238]. In experimental mouse models of RA, genetic and pharmacologically induced G-CSF deficiency prevented acute and chronic arthritis [239], possibly because it reduced myeloid cell differentiation in bone marrow.

Concluding remarks

It is becoming increasingly understood that a variety of processes controlled by the adaptive immune system are the major determinants of RA. But, adaptive immunity requires instructive signals provided by innate immune effector mechanisms, thus leaving open the question of which is/are the triggers that inadvertently fire self-reactive lymphocytes. Furthermore, even though RA does not develop without lymphocytes, disease only manifests when innate immune effector cells infiltrate articular spaces and cause pathology. Of note, the resorptive activity of synovial osteoclasts is a hallmark feature of chronic, advanced stage RA and is a major contributor to articular damage.

Considering the multiple pathways EBI2 signaling is involved in (namely osteoclast development and the generation of adaptive immune responses), which are central to the development of inflammation in articular spaces, antagonists of EBI2 signaling may provide significant protection against RA. Understanding the multitude of osteoclast differentiation pathways and mechanisms of osteoclast precursor recruitment in homeostasis and during inflammation is thus of utmost importance.

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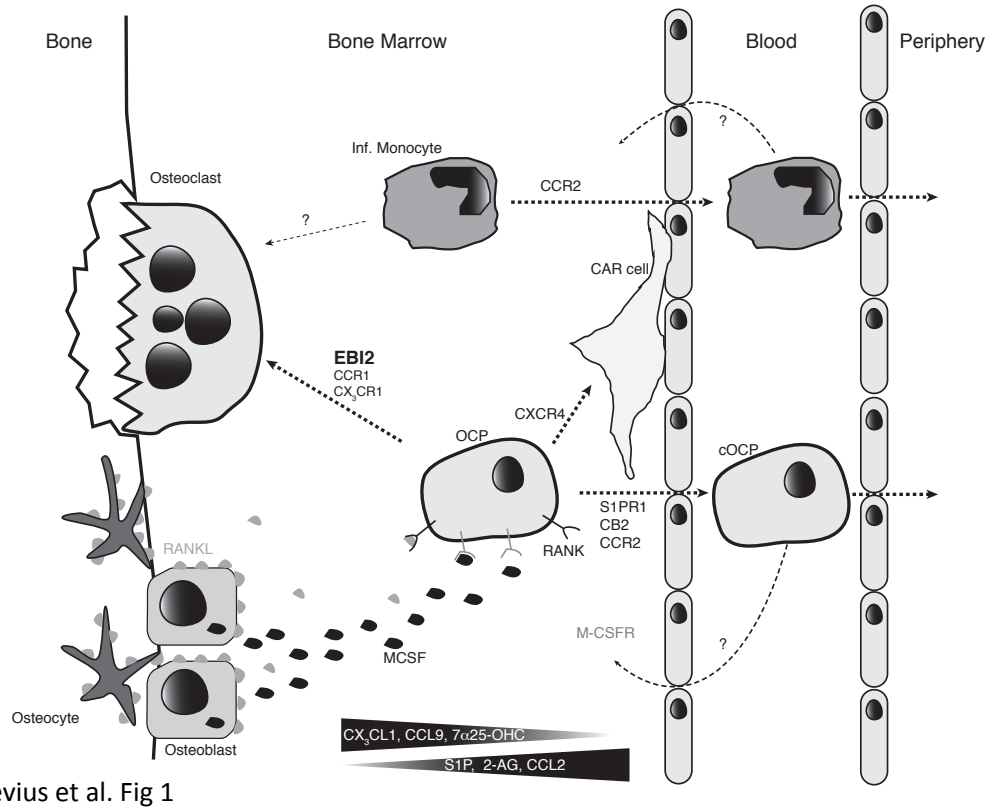
Figure legends:

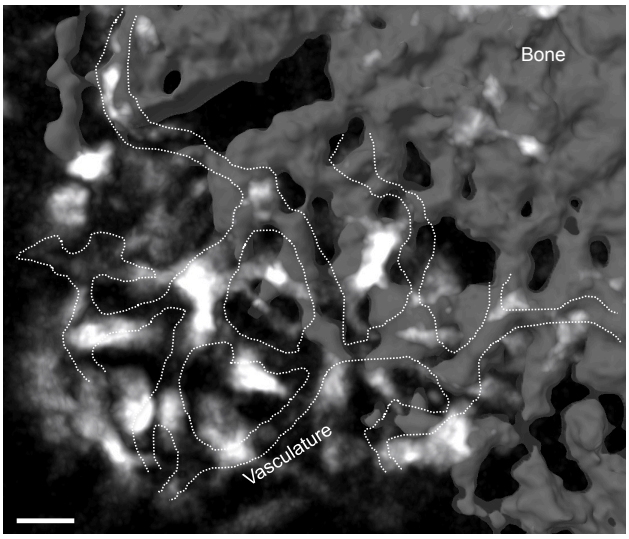
Fig. 1 Trafficking of osteoclast precursors in the bone marrow (BM), blood, and peripheral tissues. Monocytic osteoclast precursor cells (OCP) and inflammatory monocytes (Inf Mono) migrate into osteoclastogenic niches where osteoblasts (OB) and osteocytes provide important signals for osteoclast differentiation (MCSF and RANKL). EBI2 directs the migration of OCP to the endosteum, where the EBI2 ligand 7α, 25-OHC is presumably abundant. CX_3CR1 promotes the retention of OCP in BM parenchyma, and possibly directs cells to the endosteum in response to CX_3CL1 produced by OB. OB also express CCR1 ligands (e.g. CCL9) and CCR1 may also direct OCP to sites of osteoclast differentiation. S1PR1 is essential for the egress of monocytic cells in the bone marrow as they follow S1P gradients into the circulation and inflammatory monocytes egress via CCR2. CXCR4 is likely to guide OCP away from sites of osteoclastogenesis given that its ligand CXCL12 is abundant parenchymal and perivacular mesenchymal stromal cells, while it is reduced in OBs. The CB2 ligand 2-AG is likely abundant in the BM sinusoids, and may guide OCP away from osteoclastogenic niches. Both circulatory OCP and inflammatory monocytes re-enter into BM by unknown mechanisms.

Fig. 2 Osteoclasts are located in close proximity to blood vessels in the bone marrow. Image acquired using two-photon microscopy of calvaria bone marrow of $TRAP^{Red}$ reporter mouse. Osteoclasts (white); blood vessels (dashed line) were visualized with injection of dextran-FITC; and bone (gray). Scale bar= 30μm

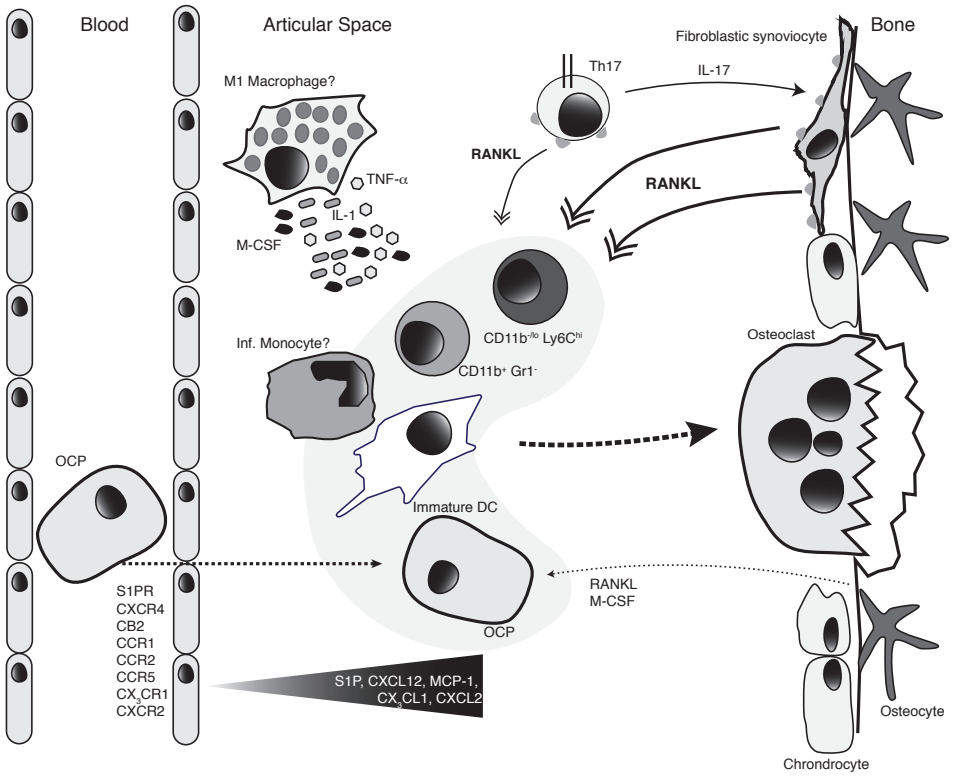
Fig. 3 Trafficking of monocytic osteoclast precursors (OCP) into inflamed joints. Cells with osteoclastogenic potential include $CD11b^{-/lo}Ly6C^{hi}$, $CD11b^{+}GR\text{-}1^{-}$, and DCs. In RA, sinusoidal fibroblastic cells provide RANKL, which can be induced by IL-17 provided by Th17 cells. The cytokines TNF-α, IL-1, and IL-6, which may be locally secreted by macrophages also promote osteoclast differentiation under inflammatory conditions. S1P receptor expression on OCPs possibly directs cells into the synovial tissue where S1P is upregulated during inflammation. CXCR4 also likely directs cells into the synovial tissue with fibroblasts, and possibly other cells, express high levels of CXCL12. Selective antagonism of CB2 inhibits the migration of monocytes into the synovium, indicating that 2-Ag levels may be present in synovial fluid. CXCR2, CX_3CR1, CCR1, CCR2, and CCR5 are also implicated in inflammatory cell recruitment into the inflamed articular space.



Nevius et al. Fig 1



Nevius et al. Fig 2



Nevius et al. Fig 3

Hematopoietic niches, Erythropoiesis and Anemia of Chronic Infection

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Abstract

Anemia is an important co-morbidity of chronic infections, as well as other inflammatory diseases. Anemia of chronic infections results from defective bone marrow erythropoiesis. Although the limitation of iron availability has been considered one key factor, the exact mechanisms underlying the blockade in erythroid generation during infection are not fully understood. Erythropoiesis is a tightly regulated process, which is very sensitive to environmental changes. During the last decade, the importance of the bone marrow hematopoietic niche has been progressively acknowledged. Several bone marrow cell types (such as Macrophages or Mesenchymal stem and progenitors cells) and molecular mediators (such as CXCL12) have been identified as fundamental for both the maintenance of hematopoietic stem cells (HSC) pluripotency and for their most adequate differentiation into each hematopoietic cell lineage. Importantly, both niche supporting cells and hematopoietic progenitors were found to be able to sense local and systemic cues in order to adapt the hematopoietic output to the organism needs. Here, we review how hematopoietic progenitors and niche supporting cells sense and respond to stress cues, and suggest a potential role for the hematopoietic niche in the development of anemia of chronic infections.

Key words: Erythropoiesis; Hematopoietic Niche; Mesenchymal Stem cells

Hematopoiesis and the Bone Marrow Microenvironment

Hematopoiesis is a critical biological process, responsible for the generation not only of the different types of leukocytes but also of erythrocytes, which have the major task of delivering oxygen to peripheral tissues. All blood cells are generated from hematopoietic stem cells (HSC), which are maintained mainly in the bone marrow (BM) during adult life. To ensure a continuous and controlled output of hematopoietic cells, some HSC lose their quiescence and enter a highly regulated

differentiation process. It has been increasingly acknowledged that HSC decision to enter a self-renewal or a differentiation process is determined by microenvironmental signals[1]. This BM microenvironment or niche in turn senses and responds to the state and needs of different tissues of the organism.

In the last decade, the cellular constituents of the hematopoietic niche started to be highlighted. Mesenchymal stem and progenitors cells (MSPC), which are multipotent cells capable of differentiating into osteoblasts, chondrocytes, adipocytes and stromal cells[2], produce factors, such as stem cell factor (SCF) and C-X-C motif ligand 12 (CXCL12), important for HSC self-renewal, survival and retention in the niche[3-14]. Despite early reports implying osteoblasts in the regulation of HSC maintenance niche[15-19], it is currently accepted that osteoblasts do not directly regulate HSC, but support B lymphopoiesis[8,17,20-22]. $CD169^+$ macrophages also regulate the hematopoietic niche by cross-talking with MSPC, inducing the expression of the chemokine CXCL12 and thus promoting the retention of HSC and progenitors in the hematopoietic niche[23]. Other mature hematopoietic cells also play a role in forming the hematopoietic niche. Megakaryocytes secrete the chemokine CXCL4, which controls HSC cell cycle activity[24]. Mature hematopoietic cells can also stimulate hematopoiesis by acting on MSPC. $CD8^+$ cytotoxic T cells (CTL), a key component of the host defense against intracellular pathogens, secret interferon gamma (IFNγ), which acts on BM MSPC, promoting their release of cytokines such as interleukin (IL)-6. IL-6 produced by MSPC leads to the increase in myelopoiesis, through the down-regulation of the transcription factors Runx-1 and Cebp alpha in early hematopoietic progenitor cells[25]. These observations suggest that mature hematopoietic cells can influence early multipotent hematopoietic progenitors, modulating their differentiation into specific hematopoietic lineages. They also imply that MSPC are important constituents of the BM microenvironment/niche, acting as regulators of hematopoietic differentiation in response to local and peripheral environmental cues. During infections, this response is based most likely on the expression of cytokine and chemokine receptors. Apart from these, hormones can also impact hematopoiesis. HSC express high amounts of estrogen receptor-alpha, whose signaling promotes HSC self-renewal, and erythropoiesis during pregnancy[26]. Additionally, central nervous system (CNS) signals also impact hematopoiesis. Sympathetic nerves enwrap MSPC and secret noradrenaline in a circadian-manner, which is sensed by MSPC and causes them to reduce the expression of CXCL12, thus enhancing the BM egress of HSC and progenitors[27,28].

Despite all the recent efforts to understand the cellular and molecular components of the HSC maintenance niche, there is still a gap in the understanding of how the niche instructs and supports specific lineage differentiation, especially during stress responses, such as infection.

Bone Marrow Erythropoiesis

Erythropoiesis occurs mostly in BM during adult life and proceeds through highly controlled sequential stages of differentiation from HSC to mature red blod cells (RBC) (see figure 1). The transcription factor PU.1 is critical for the different hematopoietic lineages fate decisions. In erythropoiesis, PU.1 sustains the self-renewal of early hematopoietic progenitor cells, preventing their differentiation in the erythroid lineage[29].

HSC as well as megakaryocytic and erythroid progenitors depend on stromal cells to be held in place through integrin-mediated adhesion and to receive instructive cytokines in order to undergo erythroid differentiation[30]. HSC and the first erythroid lineage committed progenitor, burst-forming unit-erythroid (BFU-E), are dependent on SCF (produced most likely by MSPC), whose ligand is cKIT. SCF is needed for the survival and proliferation of early erythroid progenitors and precursors up to the colony forming unit – erythroid stage (CFU-E). By preventing further erythroid differentiation and maturation, SCF signaling ensures the self-renewal of the erythroid progenitor pool. cKIT is highly expressed in BFU-E and CFU-E, but down-regulated at later stages of CFU-E differentiation. It is not expressed by polychromatic and orthochromatic erythroblasts, suggesting that the later stages of erythrocyte differentiation are mostly independent of SCF but critically dependent on erythropoietin (Epo). Epo instructs erythropoiesis in vivo by suppressing non-erythroid lineage potential, inducing an erythroid lineage bias in early hematopoietic progenitors, increasing erythropoiesis and decreasing myelopoiesis[31]. Thus, Epo is an example of a systemically-released cytokine which regulates lineage choices in multipotent hematopoietic cells present in the bone marrow. Epo is produced by cortical interstitial cells adjacent to the proximal tubules in the kidney, and is responsible for maintaining the homeostasis of circulating erythrocyte numbers. The systemic concentrations of Epo are kept low, and small decreases in the hematocrit result in exponential increases in the serum concentrations of the cytokine. Epo and SCF act in synergy to induce the proliferation of pro-erythroblasts (Pro-E), which differentiate into erythroblasts[32]. Erythroblasts surround BM macrophages, forming the erythroblastic islands dispersed throughout the BM. Within erythroblastic islands, erythroblasts proliferate, differentiate and enucleate. $CD169^+$ macrophages are a key component of erythroblastic islands in the BM, and promote late erythroid maturation[33]. As mentioned before, $CD169^+$ macrophages also cross-talk with MSPC in the BM niche, inducing the expression of the CXCL12, thus promoting the retention of HSC and progenitors in the hematopoietic niche[23]. Depletion of $CD169^+$ macrophages (for example through granulocyte colony stimulating factor (G-CSF) treatment) leads to mobilization of HSC to the blood and impaired medullary erythropoiesis[34-36]. Within the erythroblastic islands, the macrophages are thought to be involved in providing iron to erythroblasts to be used in the synthesis of heme[37]. Erythroblasts, later, expel their nuclei during nuclear extrusion, which leads to the formation of anucleated reticulocytes[38,39].

As part of the erythroblast response to Epo, erythropoietin receptor (EpoR) signaling leads to their production of erythroferrone. Circulating erythroferrone acts

on hepatocytes, causing a down-regulation of hepcidin translation. The decrease in hepcidin (see below) augments the levels of transferrin-bound iron in circulation and therefore contributes to the stimulation of erythropoiesis, in a positive feedback loop[40].

Iron homeostasis and Anemia of Chronic Infection

Iron homeostasis is tightly regulated by several mechanisms, acting either at the cellular or at the systemic levels. At a systemic level, tissue iron stores and plasma iron levels are mostly regulated by hepcidin, a small peptide hormone produced by hepatocytes. Hepcidin impacts iron distribution by binding to ferroportin and inducing its degradation. Ferroportin is an iron exporter expressed by enterocytes, hepatocytes, macrophages and adipocytes[41]. Therefore, increases in hepcidin levels decrease iron export, causing iron intracellular sequestration (mainly inside enterocytes and macrophages) and hypoferremia. Hepcidin expression is induced by two main signals: iron overload and inflammation. Increases in plasma iron concentration induce hepcidin expression in hepatocytes, by a mechanism which involves BMP/SMAD signaling and is important to avoid tissue iron toxicity[42]. During inflammation, IL-6 is mostly produced by macrophages, and induces the production of hepcidin in hepatocytes. Macrophages and adipocytes can also express low amounts of hepcidin mRNA[43-46]. Consequently, hypoferremia of inflammation develops within a few hours.

Anemia of chronic inflammation is a pathologic state characterized by impaired erythropoiesis, associated to a low concentration of circulating transferrin-bound iron as a consequence of a prolonged inflammatory state, such as chronic infection[47]. Long lasting hepcidin production and consequent hypoferremia have been postulated to be responsible for the development of anemia of inflammation. Erythropoiesis is known to be very sensitive to the transferrin-bound iron concentration[48]. It is not clear for the moment whether iron scarcity directly impairs erythropoiesis (due to unavailability of iron for incorporation in heme) or is sensed by erythroid progenitors and/or niche cells and indirectly prevents erythroid differentiation.

One possible mechanism by which iron scarcity leads to impaired erythropoiesis is through the inhibition of aconitase activity in erythroid precursors[49,50]. Aconitases catalyze the isomerization of citrate to isocitrate in the Krebs Cycle. Due to its iron-sulfur cluster, aconitase activity is iron-dependent. Aconitase activity is necessary for a correct erythropoietic process, by mechanisms involving the regulation of PU.1 expression. In situations of anemia caused by iron deprivation or by an inflammatory stimulus, the administration of isocitrate, the product of aconitase activity, reversed the anemia development[49,50].

The role of Hematopoietic niche(s) in Anemia of Chronic Infection

Although, as stated above, iron deprivation may play an important role in the development of anemia of inflammation, several previous studies suggest that hepcidin-independent pathways are also important[44,45]. Chronic mycobacterial infection induces anemia independently of the up-regulation of hepcidin[51]. Anemia is triggered by the administration of cytokines such as tumor necrosis factor (TNF) alpha, IL-1 and IFNγ in vivo[52-54]. In vitro studies have highlighted the molecular mechanisms by which IFNγ inhibits erythropoiesis. IFNγ activates IRF1 on erythroid precursors, which, in turn, induces the transcription of PU.1 and its target genes. The increase in PU.1 antagonizes GATA-1 activity, causing an increase in myelopoiesis at the expense of erythropoiesis, which is arrested.

IFNγ was also shown to induce the proliferation of HSC[55]. In response to *Mycobacterium avium* infection, IFNγ directly induces the proliferation of long-term repopulating HSC, and consequently the increase of downstream hematopoietic progenitors. These results suggest that IFNγ activates HSC proliferation to replenish immune effector cells during an ongoing infectious process. IFNα also induces HSC proliferation. Interestingly, IFNα receptor-deficient HSC were shown to proliferate in response to IFNα treatment in vivo, indicating that an indirect mechanism is involved in this effect[56]. It is possible that MSPC in the hematopoietic niche sense the inflammatory stimulus and feedback to the HSC, inducing their proliferation. A potential candidate to be modulated by MSPC is CXCL12. CXCL12 and its ligand CXCR4 have been implicated in the retention of HSC within the niche[7] and in the maintenance of HSC quiescence[11,57].

Several evidences indicate that niche-supporting non-hematopoietic cells, namely MSPC, are capable of directly responding to infection and inflammatory stimuli. Importantly, murine and human MSPC express functional Toll-like receptors (TLR), which recognize molecular patterns expressed by microbial pathogens. The activation of TLR on these cells leads to the production of inflammatory cytokines and chemokines[58-62]. Inflammatory monocytes are fundamental for defense against pathogens, and are recruited from BM to sites of infection. CCL2, a key chemokine necessary for the recruitment of inflammatory monocytes from the BM to the sites of infection, is mostly produced by hematopoietic niche-supporting MSPC[12,62,63]. MSPC respond to inflammatory cytokines as it is illustrated by the fact that the local concentration of IFNγ in the BM determines whether osteoblastogenesis[63] or adipogenesis (from MSPC) is predominantly favored, with important consequences on bone formation and functionality. In agreement with these observations is the fact that advanced osteoporosis is frequently associated with chronic inflammatory pathologies[64,65]. Also of interest, MSPC are capable of stimulating $CD4^+$ and $CD8^+$ T cells differentiation towards a regulatory cell phenotype in an IFNγ-mediated manner[66-68].

Interestingly, MSPC also express heme oxygenase 1 (HO1), which is fundamental for MSPC immune-modulatory activity[69]. HO-1 expressed by MSPC induces the differentiation of T cells into specific T regulatory subsets as well as IL-10

production[70], which, in turn, may act on dendritic precursor cells changing their fate and inducing the acquisition of regulatory functions[71]. HO1 is similarly crucial in erythroblastic island macrophages for the generation of new erythrocytes, as deficiency in HO1 leads to the depletion of blood island macrophages most likely due to toxic effects caused by the accumulation of non-catalyzed heme[72]. Of note, HO-1 is up-regulated during mycobacteria-induced anemia[51] and its expression in macrophages is crucial for resistance to mycobacteria chronic infections[73].

All these observations support the hypothesis that systemic chronic inflammation, namely associated with infection, may cause alterations within the MSPC microenvironment, which may consequently alter the erythrotopoietic output.

The erythropoiesis regulation triangle

BM erythroid progenitors, BM niche cells (e.g. MSPC and macrophages) and peripheral signals crosstalk to maintain an optimal but controlled output of RBC (Figure 2). During infection, inflammatory signals are sensed by hepatocytes, which release hepcidin. Hepcidin in turn acts mostly on enterotocytes and macrophages to sequester iron, decreasing the amount of serum iron available for erythropoiesis. Erythroid progenitors sense Epo and are instructed to the erythroid fate and feedback to the hepatocytes by producing erythroferrone, which will suppress hepcidin hence enhancing erythropoiesis. BM MSPC and macrophages are the third corner of this triangle. With their capacity to sense inflammatory stimuli and respond to them with the release of several mediators, these cells have a strong impact on the fate of multipotent hematopoietic progenitors, namely, instructing them towards some hematopoietic lineages at the expense of others. The mechanisms by which the hematopoietic niche regulates the erythrocyte output are still poorly characterized. Inflammatory stimuli decrease the amount of CXCL12 and increase the concentration of G-CSF, reducing the numbers of $CD169^+$ macrophages, and increasing the proliferation and mobilization of HSPC. The lack of $CD169^+$ macrophages feedbacks by mobilizing more HSC and preventing the formation of erythroblastic islands. Of note, TNF-α, one of the cytokines overproduced during chronic infections, is a known inhibitor of erythropoiesis[74]. In fact, both MSPC and HSPC can sense the inflammatory cytokines and cross talk to adjust their fate. HSPC will differentiate mostly into myeloid cells at the expense of lymphopoiesis or erythropoiesis. Simultaneously, the inflammatory milieu may favor the differentiation of MSPC into adipocytes, which have been described as negative regulators of hematopoietic activity[75], and expressing low amounts of hepcidin mRNA. An increase in adipocytes in the BM may contribute to a local increase in hepcidin levels in the BM. Increased hepcidin levels in BM together with systemic hepcidin-induced hypoferremia may decrease iron availability for aconitase activity, heme synthesis, and erythropoiesis in general.

A deeper understanding of the regulation of the BM microenvironment and its crosstalk with HSPC to guarantee the best hematopoietic output, either in

homeostasis or during stress responses, is clearly needed. Anemia of chronic inflammation is an important co-morbidity of several diseases, representing an additional challenge in terms of therapeutic options. Filling the gaps in understanding the complex networks (cell types and molecular mediators) that enable hematopoietic differentiation and lineage choice will enhance the clinical options to deal with anemia associated with pathologies such chronic infections or leukemia.

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Figure 1. Developmental progression of erythroid lineage from HSC to mature erythrocytes. The most important factors involved in specific developmental stages are depicted.

Figure 2. The erythropoiesis regulation triangle: crosstalk between MSPC, erythroblastic islands macrophages and HSPC during response to systemic signals. Changes in systemic signals occur during infection and are sensed by HSPC, MSPC and macrophages in BM parenchyma. MSPC and macrophages produce constitutive factors that act on HSPC instructing their self-renewal and/or lineage differentiation towards specific lineages. Systemic signals such as inflammatory cytokines can also modulate the production of these factors and, consequently, HSPC are instructed to specific hematopoietic lineages (e.g. myeloid) at the expense of others (e.g. lymphoid and erythroid). Chronic exposure to inflammatory systemic signals triggers a blockade on erythropoiesis and subsequently the development of anemia.

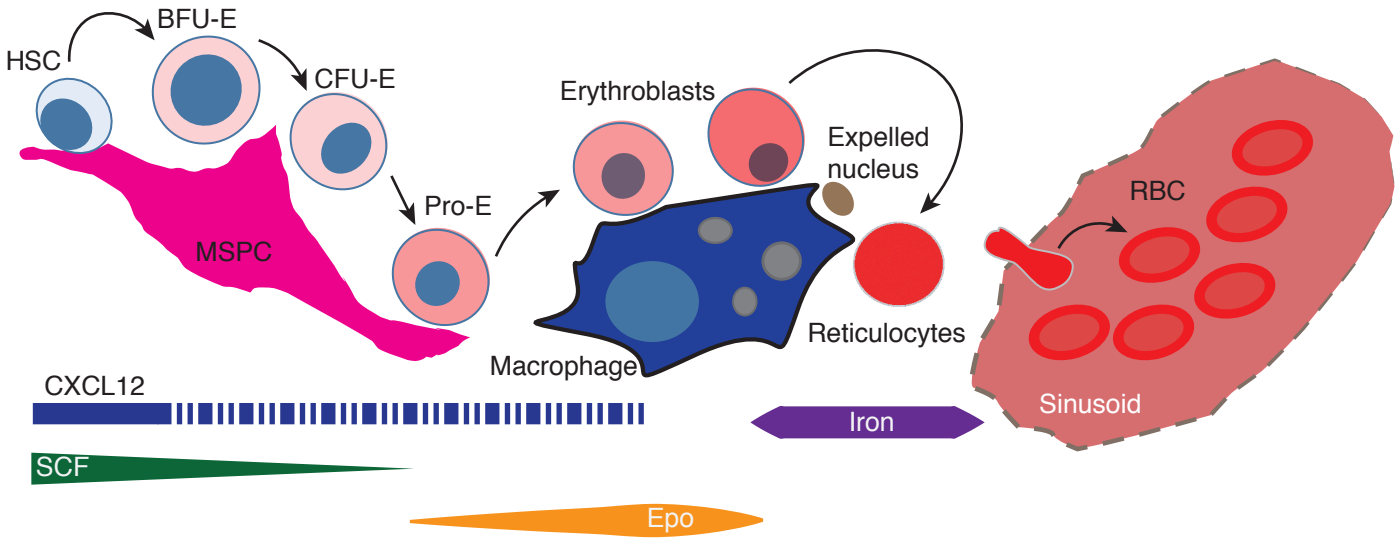
HSC
BFU-E
CFU-E
Pro-E
MSPC
Erythroblasts
Expelled
nucleus
Macrophage
Reticulocytes
RBC
Sinusoid
CXCL12
Iron
SCF
Epo

Figure 1

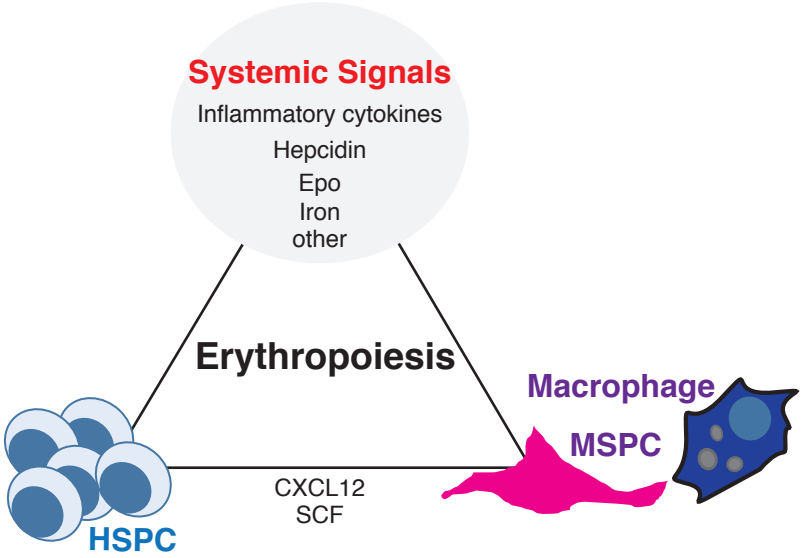
Systemic Signals
Inflammatory cytokines
Hepcidin
Epo
Iron
other
Erythropoiesis
Macrophage
MSPC
CXCL12
SCF
HSPC

Figure 2